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ANTIVIRAL ACTIVITIES OF SUPERNATANT OF FERMENTED MAIZE *(OMIDUN)* AGAINST SELECTED ENTEROVIRUSES

[1]Sunmola, A. A., [*2]Ogbole, O. O., [3]Faleye, T. O. C., [2]Adeniji, J. A. and [*1]Ayeni, F. A.

[1]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Ibadan, Oyo State, Nigeria
[2]Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Oyo State, Nigeria
[3]Department of Virology, University College Hospital, University of Ibadan, Ibadan, Oyo State, Nigeria

[*]Corresponding authors email: nikeoa@yahoo.com, funmiyeni@yahoo.co.uk

ABSTRACT

Enteroviruses (EVs) are of enormous public health significance being the etiological agents of an array of clinical conditions, local fermented product may confer protection in the gastrointestinal tract against EVs. *Omidun* (supernatant of fermented maize) has been traditionally used to reduce the frequency of stooling during diarrheal episodes. However, there is no information on the antiviral activities of *Omidun* on EVs and the scientific proof of the traditional claims. This study, therefore, investigates the antiviral potentials of *Omidun* against EVs. The antiviral activity of *Omidun* was determined against Echovirus 7 (E7), E13 and E19 in a pre- and post-treatment approach. The antiviral effect was determined by cytopathic effect and cell viability using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor. (MTT) assay. *Omidun* had high percentage inhibition for E7 and E19 with 83.5% and 77.9% respectively, in pre-treatment, and 78.2% and 77.5% respectively, in post-treatment. Minimal effects were observed for E13; 12.2% and 24.2% in pre- and post-treatment, respectively. Generally, antiviral activity for pre-treatment was better than post-treatment. *Omidun* was shown to have the potential to inhibit the replication of selected EVs. Thus, supporting the traditional claims of its therapeutic effect. To the best of our knowledge, this is the first report of antiviral activities of *Omidun*.

Keywords: *Omidun*, Human Enterovirus and Antiviral

INTRODUCTION

Enterovirus (EV) is a genus in the *Picornaviridae* family (Tan *et al*., 2011) and poliovirus (the etiological agent of poliomyelitis) is the prototype members of the genus. The elimination of poliovirus through vaccination in many parts of the world and the ultimate eradication (in the near future) of the virus globally has resulted in non-polio EVs (NPEV) taking center stage as agents of public health significance. This is manifest in the emergence of EV-D68 and EV-A71 as the leading etiological agents of EV associated neurological manifestations like Acute Flaccid Myelitis (AFM); a clinical condition very similar to poliomyelitis and seems to be taking its niche) globally (Bitnun *et al*.,2018). Considering the public health significance of NPEVs and the dearth of vaccines and chemotherapeutics agents, there is need for investigation of alternative strategies for NPEV control.

Enteroviruses are spread mainly via the faecal-oral route and the gastrointestinal tract serves as their site of primary replication (Nathanson *et al*., 2010; Tapparel *et al*., 2013). Lactic acid bacteria (LAB) are very effective in the treatment of gastrointestinal diseases (Pant *et al*., 2007; Bamidele *et al.* 2013; Bamidele *et al.* 2014; Sunmola *et al*. 2019) and they naturally reside in fermented foods. In Nigeria, one very common indigenous fermented food with functional lactic acid bacteria is O*gi*. It is an acidic fermented cereal mash formulated from maize. It is usually prepared as a smooth porridge with sour taste and redolent similar to yoghurt (Falana *et al*., 2011) and serve as staple meal for convalescences and the aged as well as popular weaning food for infants [Teniola and Odunfa 2001; Omemu *et al*., 2011]. *Omidun* is the watery supernatant of "*Ogi*" and has been traditionally found to be of medicinal importance in the South-Western part of Nigeria. It is used as solvent for herbal extraction especially to treat malaria and fevers (Falana *et al*., 2016). *Omidun* is also used traditionally for the control of diarrhoea and other GIT disorders ([Teniola and Odunfa .2001). Microorganisms, especially LAB in *Omidun* and other indigenous fermented foods have been reported to have antimicrobial efficacy against pathogenic microorganisms (Ayeni *et al*., 2006; Ayeni *et al*., 2009; Ayeni *et al*., 2011; Falana *et al*., 2012; Afolayan and Ayeni 2017). However, in spite of traditional claims of the therapeutic efficacy of *Omidun* against GIT disorders, there is no report of antiviral activities of *Omidun* against EVs. Thus, in this study, we aim to examine the *in vitro* antiviral activity of *Omidun* against three NPEVs (Echovirus 7 [E7], E13, and E19) commonly recovered from acute flaccid paralysis cases in Nigeria

METHODOLOGY

Cultures of cell line

Human rhabdomyosarcoma (RD) cells were maintained in Dulbecco's modified eagle media (DMEM) supplemented with 10 percentage v/v heat-inactivated foetal bovine serum (FBS). Cells were detached from flask (trypsinized) using 0.25% v/v trypsin. Subsequently, growth medium (DMEM supplemented with 10% FBS) was used to terminate trypsinization and 100 µL of medium with cells were

transferred into 96 wells plate, then, incubated in humidified incubator at 37ºC, 5% Carbon dioxide for 24 hrs. The cells used were passaged between 7 to 10 times throughout the study.

Viruses

Three echoviruses (E7, E13 and E19) previously recovered from acute flaccid paralysis cases (Faleye *et al*., 2017) were passaged in RD cell line (UCH-WHO group code RD/P231). They were freeze-thawed three times, centrifuged and the supernatant was aliquoted into 500-µl cryovials and stored at − 20 C until use.

Virus quantification and infection titre calculation

A method similar to that used by (Wei *et al*., (2014) was used to quantify the Echoviruses used in this study. One hundred microliters (100 µL) of RD cell suspension was seeded into each well of a 96 well plates as previously described. After 24 hours incubation, 100 µl of 10-fold serially diluted viral suspension was added and then incubated till the virus control wells had developed 100% cytopathic effects (CPE) after 24 h. The CPE were scored visually using an inverted microscope and the virus infection titers were expressed as Tissue Culture Infective Dose ($TCID_{50}$). Viral titers were determined using the standard method of median tissue culture infective dose ($TCID_{50}$) on the cells. Infectious titer was calculated using Kerber Spearmans formular.

Preparation of *Omidun*

Fresh *Ogi* was prepared as previously described by Afolayan *et al.* (2017). The whole mass was re-suspended in water and allowed to ferment for 24 hours. The supernatant (*Omidun*) was obtained. The maximum number of bacteria (Minimum Cytotoxic Concentration; MCC) introduced into the antiviral assays was determined by titration (cells exposed to different *Omidun* concentrations) against the cell line; a process similar to cell cytotoxic concentration (CC_{50}) by Wei *et al*., (2014). The MCC was carried out by preparing twelve (12) 1:2 serial dilutions of the *Omidun*. Each dilution was inoculated (in eight wells) into the monolayer of cells in 96 well plate. Dilution 11, found to be the dilution with the least viable cells, was plated out on de Mann Rogosa Sharpe (MRS) agar to confirm presence of LAB which gave a count of 36.

Antiviral effect: Pre treatment

The antiviral activities of *Omidun* against E7, E13 and E19 was determined according to inhibition of virus-induced CPE in acutely infected RD cells. Confluent RD cell monolayer in 96 well plate was inoculated with 50 µL of *Omidun* in triplicates and allow to adsorb for 90 mins after which 50 µL of the respective viruses (E7, E13 and E19) containing 100 $TCID_{50}$ were added (Khania *et al*., 2012). The preparation was sealed and incubated in humidified incubator at 37ºC, 5% carbon dioxide for 48 hrs. CPE was observed microscopically and the viability of the cells was determined using MTT assays. .

Antiviral effect: Post treatment

The antiviral activities *Omidun* against E7, E13 and E19 was determined according to inhibition of virus-induced CPE in acutely infected RD cells. Confluent RD cell monolayer in 96 well plates was infected with 50 µL of virus (E7, E13 and E19) containing 100 $TCID_{50}$ and allowed to adsorb for 40 mins for pretreatment and 40 minutes for post treatment after which 50 µL of *Omidun* was added (Khania *et al*., 2012). The preparation was sealed and incubated in humidified incubator at 37ºC, 5% carbon dioxide for 48 hours. The CPE was observed microscopically and the viability of the cells was determined using MTT assays.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor (MTT) assay

The MTT assay (Sigma – USA) was used to quantify living cells via mitochondrial metabolism. The assay was carried out according to slightly modified method of Khania *et al*.,(2012). After the incubation period (48 h at 37°C) in presence of 5% carbon dioxide on a 96-well plate, the medium was removed and 25 µl of ready MTT dye was added. The plate was incubated at 37°C for 4 hrs. After this period, 100 µl of dimethyl sulphoxide (DMSO) was added; the contents were shaken for few minutes and the optical density of each well was determined with a microplate reader (Bio-Tek USA) at 490 nm.

Data analysis

The percentage inhibition was calculated with the mean optical density using Microsoft Excel software with the formula below;

$$\frac{\text{Virus control} - \text{Test}}{\text{Virus control}} \times 100\%$$

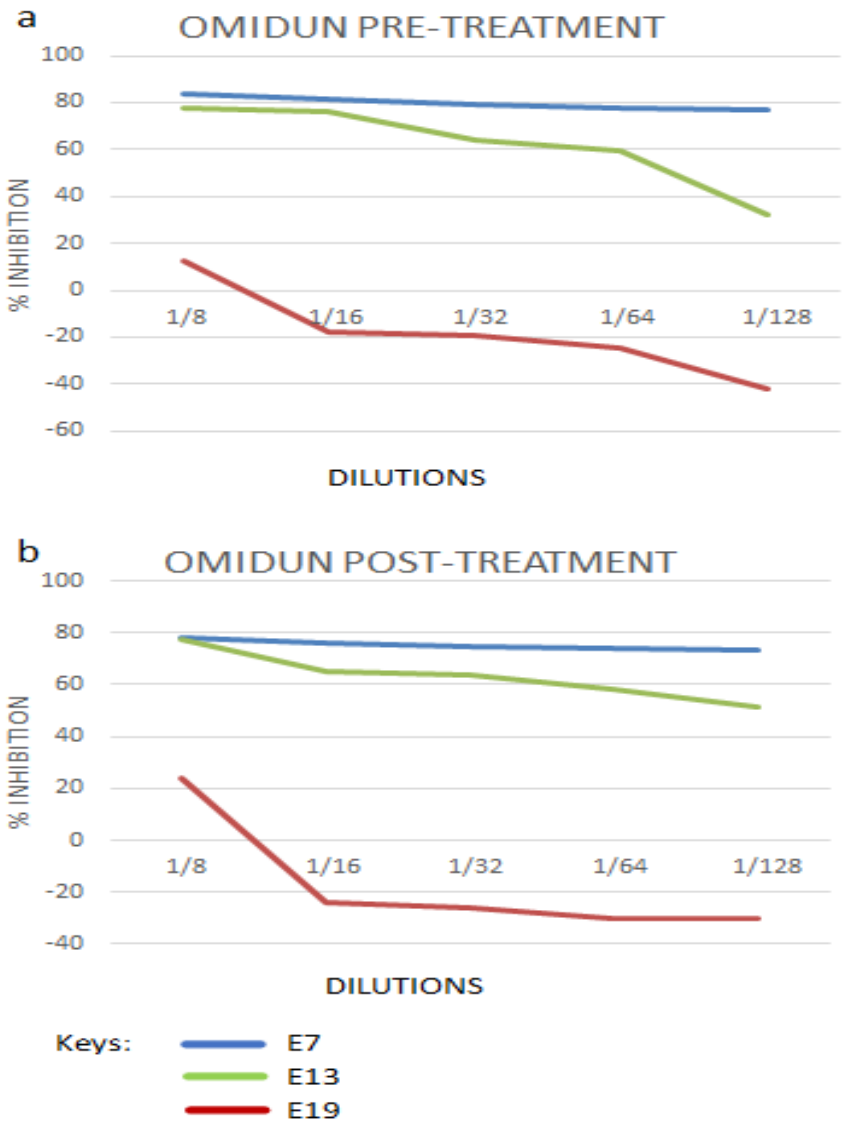
Optical densities of the MTT assay of the treatment and control wells *in vitro* anti-viral study were obtained and all results were expressed as means ± SD (Standard deviation of mean). Differences between means across groups were tested for statistical significance using a one-way analysis of variance (ANOVA) with the Tukey post hoc test. All statistical analyses were carried out with Graph Pad Prism version 5.0.

RESULTS

Antiviral activities of *Omidun* against E7, E13 and E19

Omidun was administered to the cells before (pre-treatment) infection with E7, E13 and E19 (Figure 1a), The final concentration of *Omidun* used that had best cell survival was at 1.8 x 10^2 CFU/ml of viable bacterial cell. *Omidun* had a high percentage inhibition of 83.5% and 77.9% for E7 and E19, respectively. For E13, on the other hand, the percentage inhibition was low with a value of 12.2%.

When *Omidun* was administered to the cells after (post-treatment) infection with E7, E13 and E19 (Figure 1b), *Omidun* had a percentage inhibition of 78.2%, 77.5% and 24.2% for E7, E19 and E13 respectively. As with pre-treatment, the inhibitory effects observed were most pronounced in the highest concentration of *Omidun* used.



Figures 1a and Ib: Percentage inhibition of *Omidun* for pre-treatment (a) and post-treatment (b) against EV7, EV13 and EV19 at different dilutions.

Comparison of Pre- and Post-treatment of E7, E13 and E19 infected cells with *Omidun*

For E7 and E19, the proportion of viable cells was higher in pre-treatment than post-treatment (Figure 2). The difference was however, only statistically significant ($P<0.01$) for E7. For E13, the proportion of viable cells was higher in post-treatment than pre-treatment. However, there was no statistically significant difference between pre- and post-treatment with *Omidun* against E13 and E19 (Figure 2).

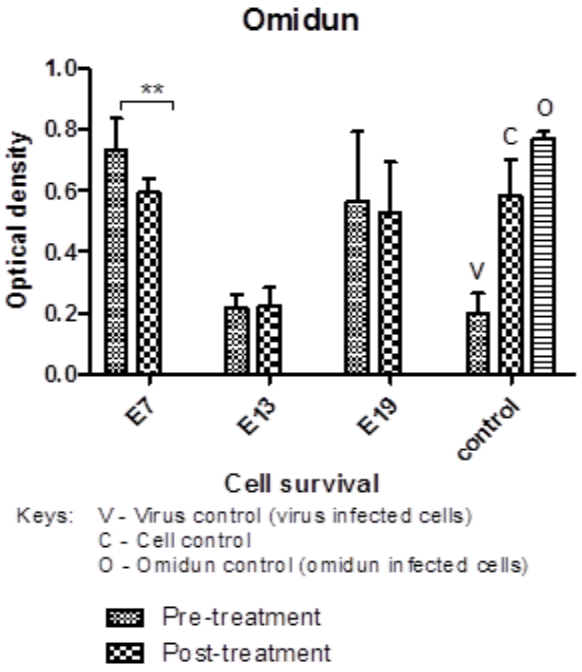


Fig. 2: Pre-treatment and post-treatment intervention of *Omidun* against EV7, EV13 and EV19 infected RD cells. Data presented as Mean ± SD <P0.01.

DISCUSSION

In this study, we have demonstrated the antiviral activity of *Omidun* against selected EVs in vitro. *Omidun* being a vehicle for LAB in fermentation state tend to potentiate its activity. Lactic acid bacteria have multiple antimicrobial mechanisms which prevent viral infection of the cells and some LAB strains have antiviral activities which deduce a strain dependent antiviral effect (Khania *et al*., 2012).

Pre-incubation approach has also been previously reported to be better than co-incubation in prevention of pathogen adherence due to the fact that some organisms adhere rapidly to cells than others and any detached LAB strains are readily replaced by surrounding pathogenic organisms (Sayyed *et al*., 2013). In this study, pre-treatment was seen to be more effective than post-treatment as displayed by *Omidun* against E7. There was no significant difference between the pre-treatment and post-treatment effect of *Omidun* used in this study for E13 and E19. The replication cycle of EVs is usually complete in six (6) hours (Carter *et al*., 2007). Hence, the 40 minutes delay in the post-infection administration of *Omidun* (post-infection assay) should have given the viruses a head start in the infection process and possibly attachment and entry should have been initiated (if not completed) by the time *Omidun* was administered. Hence, the observed antiviral activity of *Omidun* when administered post-infection might imply that a mechanism also exists by which some component of *Omidun* can halt or interrupt (therapeutically) an already initiated EV (at least E7 and E19) replication cycle. Considering, E7, E13 and E19 are all members of EV Species B (EV-B) and were recovered from AFP cases around the same time (Faleye *et al*., 2017), the well documented recombination within the non-structural genomic regions (P2 and P3) of members of the same EV Species (Nikolaidis *et al*., 2019) suggests that region of their genomes would be very similar. Hence, might not be completely responsible for the observed resistance of E13 to *Omidun* while E7 and E13 were susceptible. The switching (via recombination) of the non-structural genomic regions (P2 and P3), restores pathogenicity and transmissibility to the Sabin (Vaccine) strains of the Polioviruses and thereby contribute to the emergence of Vaccine Derived Polioviruses (Burns *et al*., 2014), It may however be difficult to completely rule out the possibility that differences in the non-structural genomic regions (P2 and P3) of the viruses could contribute to the different phenotypes observed in response to therapeutic treatment of infected cells with *Omidun.*

The control wells containing only RD cells and *Omidun* had higher values for the viability (MTT) assay compared to the control wells that had only RD cells. This suggests that some components of *Omidun* might be helping the cells grow better or remain viable longer. The other possibility is that some of the LAB in the *Omidun* could have been present in the wells during the MTT assay and (being viable cells too) increased the read-out for viability (Hundie *et al*., 2016). Considering that these LAB might have been present in the experiment wells containing RD cells, EVs and *Omidun*, it is likely that the values for the viability (MTT) assay might have been exaggerated by measuring both viable RD and LAB.

CONCLUSION

Omidun, was able to reduce the viral infectivity of the Enterovirus-7 and Enterovirus -19 to a large extent and its pre-treatment is more effective than its post-treatment. Constant consumption of *Omidun* might be beneficial for its antiviral effects.

LIMITATION OF THE STUDY

The viable RD cells were not delineate from viable lactic acid bacteria that might be present in *Omidun.*

COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

AAS carried out laboratory studies and drafted the manuscript. OOO designed the study and revised the manuscript. TOCF revised the manuscript. JAA provided the viral materials for the study, FAA designed the study and revised the manuscript.

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