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Treatment Effect of Various Concentration of Plant Extracts on Murine Norovirus

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Abstract

Noroviruses are positive-sense, single-stranded, non-enveloped RNA virus that measures approximately 27 - 35 nm in diameter. It affects humans of all ages and races causing most cases of viral gastroenteritis worldwide. Infection results from ingestion of contaminated food or water as well as causing diarrhea and vomiting in humans. Extracts from plants are known to have antioxidant, anti-inflammatory, and adhesive properties which are associated with barrier functions. The aim of this study was to elucidate whether plaque reduction was due to an effect of methanolic plant extract directly on the virus, whether the extract affects viral replication, and lastly, whether the extract disrupts the cell surface binding with the virus. The plant extracts of interest were the calyces of *Hibiscus sabdariffa* (HS) and the seeds of *Zanthoxylum armatum* (ZA). Antiviral activities of these extracts were determined against murine norovirus. The logarithmic viral reduction per plaque-forming unit was (22 $\log_{10}$) PFU/ml (control), (15 $\log_{10}$) PFU/ml (treated HS), and (12 $\log_{10}$) PFU/ml (treated ZA) with a significant reduction (68% and 55% respectively) when compared with the control for the direct effect on the virus. The role of extracts on virus replication showed (25 $\log_{10}$) PFU/ml (control) as against the HS treated-virus-infected cells (9 $\log_{10}$) PFU/ml and ZA treated-virus-infected cells (5 $\log_{10}$) PFU/ml (36% and 20% respectively). Finally, effect of the extract on the viral attachment showed (31 $\log_{10}$) PFU/ml (control), (12 $\log_{10}$) (HS-treated) and (9 $\log_{10}$) PFU/ml (ZA-treated), (39% and 29% respectively. Extract treatment with HS and ZA has shown evidence of a reduced number of plaques formation with the latter having fewer plaques. Both extracts have proven potential to reduce the viral multiplication process by interfering with the replication process. This study shows that *Hibiscus sabdariffa* (calyces) and *Zanthoxylum armatum* (seed) extracts disrupt murine norovirus from consistent viral replication.

Keywords

Norovirus, *Zanthoxylum Armatum*, Hibiscus Sabdariffa, Plant Extracts, Plaque Formation, Viral Reduction

1. Introduction

Human norovirus (HuNoV) infection is transmitted through contaminated food or water, as well as infected surfaces or persons [1]. Outbreaks are rampant in the military, nursing homes, and cruise ships [2]. The HuNoV is sporadic [3], and is known to cause approximately 75% - 90% of nonbacterial gastroenteritis [4], with vomiting and diarrhea as its earliest symptoms [5]. Each year, approximately 685 million cases occur worldwide, and approximately 21 million cases occur annually in the US alone. Nearly 71,000 hospitalizations, 800 deaths, and $493 M of economic loss are accrued because of norovirus infection per year in the USA [6], with most outbreaks occurring in winter [7].

Viral diseases continue to pose a serious threat to public health. An outbreak of pneumonia with an unknown etiology emerged in China in 2019, and later, it was confirmed to be a new coronavirus and named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Recently, this severe acute respiratory syndrome was named coronavirus disease 2019 (COVID-19), and the World Health Organization declared its spread a global pandemic (8). The world has witnessed many viral epidemics in the past like severe acute respiratory syndrome coronavirus (SARS-CoV-1), H1N1 influenza, and the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2003, 2009, and 2012 respectively [8]. However, COVID-19 proves to be especially infectious. COVID-19 is a single-stranded, positive-sense RNA virus with a diameter of 60 - 140 nm and a round or elliptic shape. It belongs to the beta-coronavirus group, sharing ancestry with bat coronavirus HKU9-1, similar to SARS-coronaviruses [9]. Still, more information on its transmission is needed to control the spread.

Although the control of this viral infection is presently only through practices such as constant handwashing, confinement of infected persons, and drinking plenty of fluids, there is a need for improving treatment and prevention of norovirus infections. Plants are natural resources that have been proven to be an effective treatment against viruses with little to no side effects [10]. Plants contain phytochemicals that naturally protect them from microbe and insect attacks [11]. These phytochemicals have been found effective against bacterial infections by several mechanisms which include inhibition of the activity of toxins and enzymes destruction of virulence factors and damage to the bacterial membrane [12].

Phytochemicals, typically flavonoids, polyphenols, and organic acids are known to have therapeutic potential for most human ailments [13]. Flavonoids block prostaglandin synthesis, cell cycle progression, and protect the cell against

injury caused by X-rays [14]. Polyphenol and organic acids are known to regulate enzyme activities and proliferation of bacteria respectively. The plants of interest in this study were *Hibiscus sabdariffa* (HS) and *Zanthoxylum armatum* (ZA) which are known to possess flavonoids, polyphenols, and organic acids. Studies have shown that saponins from *Zanthoxylum armatum* were effective against human breast cancer cell lines (MCF-7, MDA-MB-468) and colorectal cancer cells (Caco-2) [15], and have antiviral properties [16]. *Hibiscus sabdariffa* has a wide range of medicinal uses which include treatment of high blood pressure, urinary tract infection, colds, conjunctivitis, antiscorbutic, fluid retention, fevers, antibacterial [17], anticancer, and gastrointestinal disorder [18]. Viral plaques are products of viral growth as a result of structural changes in a host cell. The presence of this growth is called cytopathic effects (lysis of host cell) and it is usually used to count infectious particles [19]. The occurrence of plaque reduction by *Hibiscus sabdariffa* (HS) and *Zanthoxylum armatum* (ZA) extracts has never been reported. The optimal effects of these extracts on virus reduction are worth studying. This study sought to elucidate whether plaque reduction was due to an effect of methanolic plant extract directly on the virus, whether the extract affects viral replication, and or, whether the extract disrupts the cell surface binding with the virus. Insight into the finding of the direct effect of the extract on the virus is a prelude to studying the mechanism of action of these extracts on murine norovirus and developing therapeutics for norovirus.

2. Materials and Methods

Leaves of Hibiscus Sabdariffa (HS) were collected from a local farm located in Greensboro, North Carolina, USA. Leaves were washed to remove dust and freeze-dried before extraction with methanol. Whereas seeds of *Zanthoxylum armatum* were imported from Kathmandu, Nepal. Seeds of *Zanthoxylum armatum* were washed and freeze-dried before extraction with methanol. Methanolic extraction of both plants was used in this experiment as a crude extract. In this experiment, methanol extracts of HS and ZA were used in the 10% concentration against the human norovirus surrogate, the murine norovirus. The outcomes of this experiment were compared with control (untreated with plant extract).

Viral plaque reduction

Three different experimental protocols were examined to help find out the cause of plaque reduction: The protocols include: 1) To confirm if a reduction in plaques was due to an effect of the extract directly on the virus and not the cells. 2) To test whether the extract influences virus replication and 3) To test whether the extract influences the cell surface attachment. The conditions of each of the protocols are discussed below.

To confirm if a reduction in plaques were due to an effect of the extract directly on the virus and not the cells. A 100 ml of the virus was placed separately in two different sterilized 1.5 ml vials. Crude extracts of both plants of concentration 0.3 mg/ml diluted 1:5 with DMEM-20. Further, 100 ml of each of

the diluted extracts were mixed with the murine norovirus previously placed in the vials respectively and incubated in a humidified 37˚C in a 5% CO_2 incubator (Thermo Scientific NAPCO Series 8000 DH) for three hours. Diluted virus-extracts solution (200 ml) added to exponentially growing 1.41×10^7 cells/ml RAW 264.7 cells (ATCC TIB-71) (NB: media removed from the cell before adding the virus-extract solution), followed by incubation for 2 hours at 37˚C in 5% CO_2 incubator. Sea plaque agarose (3 g) dissolved in 100 ml of PBS; proper dissolution enhanced with the aid of a microwave, avoiding bumping, and then cooled to 42˚C using a water bath (To avoid potential cell death due to exposure to high temperature). Dissolved agarose in combination with fetal bovine serum (20%) 2 × MEM (2 ml) was (1:1), were used in covering the plates. The agarose and fetal bovine serum were allowed to gel for 5 minutes under a biosafety cabinet. The plates were incubated for about six days. Note that control consists of untreated virus-cell complex (Figure 1 & Figure 2).

Role of the extract on virus replication. The above protocol was followed with an absence of rinsing of the extracts. We covered the extracts-treated virus-infected cells with Sea plaque agarose and incubated them for six days to enable plaque formation (Figure 3 & Figure 4). The extract concentration remained at 0.30% in all three experiments. We used plaque reductions in quantifying the studies. Each of the experiments was performed in triplicates. Controls received no extracts.

To test whether the extract influences the cell surface attachment. This was done according to a previous method [20] with some modifications. Briefly, a 200 µl of 1:10 diluted extract was placed on the exponentially 1.41×10^7 cells/ml growing RAW 264.7 cell line (Note that the 1 in 10 dissolutions were done with complete media). The combination of extract and cells was incubated for one hour at 37˚C in 5% CO_2 in an incubator. At the end of 1hr incubation, the mixture was rinsed with media, spun down to remove free extracts, and infected with 500 µl of the virus. Then, covered with agar as above and incubated for six days (Figure 5 & Figure 6).

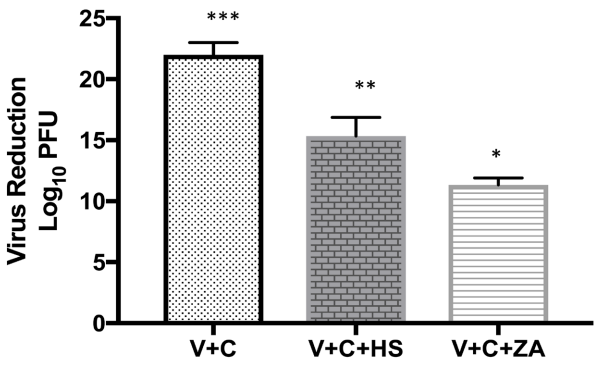

Figure 1. Cells treated with HS and ZA show viral plaque reduction. Control had no extracts (V + C), *Hibiscus sabdariffa* extract treated (V + C + HS), and *Zanthoxylum armatum* treated (V + C + ZA). Statistical significance ($p < 0.05$).

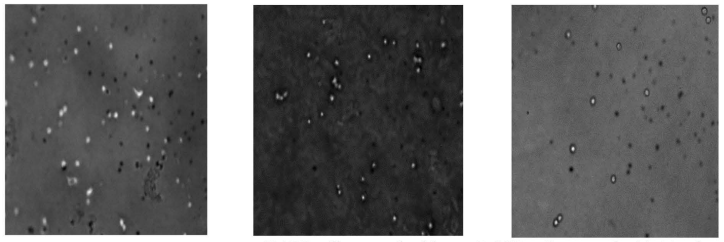

Figure 2. Micrographic representations of viral plaque reduction due to an effect of the extract directly on the virus. These were examined using "Axio Observer" inverted microscope.

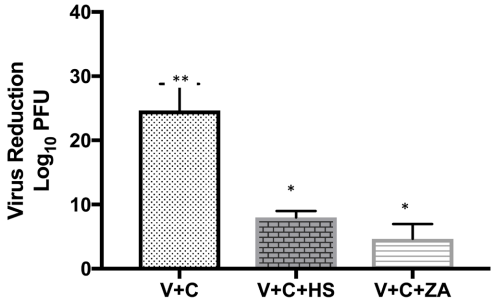

Figure 3. Influence of extracts on viral replication. Control had no extracts (V = Virus + C = cell line), *Hibiscus sabdariffa* extract treated (V + C + HS), and *Zanthoxylum armatum* treated (V + C + ZA). There are statistical significances between the tests and control ($p < 0.05$).

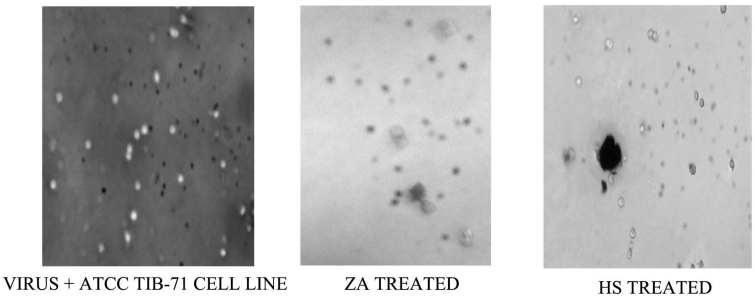

Figure 4. Micrographic images of extract influence on viral replication using "Axio Observer" inverted microscope.

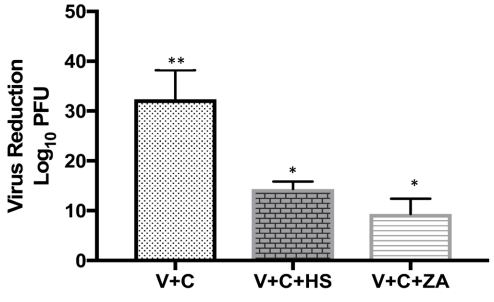

Figure 5. Extracts influence viral attachment. Control had no extracts (V + C), *Hibiscus sabdariffa* extract treated (V + C + HS), and *Zanthoxylum armatum* treated (V + C + ZA). There are statistical significances between the tests and control ($p < 0.05$).

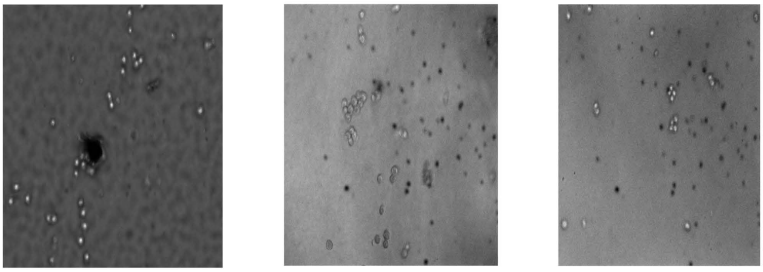

Figure 6. Micrographic representations of extracts influence on cell attachment using "Axio Observer" inverted microscope.

3. Statistical Analysis

Data are shown as means ± standard error (SE). t-test and one-way ANOVA were performed to compare means. All statistical analyses were performed using Graph-Pad Prism version 7. Differences of $p < 0.05$ were considered significant.

4. Results

Two plant extracts *Hibiscus sabdariffa* and *Zanthoxylum armatum* were investigated to evaluate whether their antiviral potentials were due to an effect of methanolic plant extract directly on the virus, whether the extract affects viral replication, or whether the extract disrupts the cell surface binding with the virus.

To confirm if a reduction in plaques formation were due to an effect of the extract directly on the virus. The outcomes of this experiment showed that extracts of both plants had some plaque reductions compared with the control. The logarithmic viral reduction per plaque-forming unit was 22 (PFU/ml) (control), 15 PFU/ml (treated HS), and 12 PFU/ml (treated ZA) (**Figure 1**) with significant reduction at the level ($p < 0.05$) when compared to the control.

To find out how the extracts influence viral replication. Further diluted extracts were placed with 1.41×10^7 cells/ml ATCC TIB-71 cell lines, incubated for an hour, rinsed, spun down to remove free extracts, and then sealed with agarose, then incubated the plates for six days. The results of this experiment for the control showed (25 $\log_{10}$) PFU as against the HS treated-virus-infected cells (9 $\log_{10}$) PFU/ml and ZA treated-virus-infected cells (5 $\log_{10}$) PFU/ml (**Figure 3**). The effect of the extract on viral attachment showed a significant reduction at the level ($p < 0.05$) among the control and tests. For the effect of the extract on the viral attachment, the control showed (31 $\log_{10}$) PFU/ml, (12 $\log_{10}$) (HS-treated), and (9 $\log_{10}$) PFU/ml (ZA-treated) (**Figure 4**). Compared to the control and the treated, there is clear evidence of viral reductions; with the ZA-treated cells having a higher number of viral reductions than the HS-treated. There were significant reductions ($p < 0.05$) among the two extracts in all the experiments compared to control at the ($p < 0.05$) level.

The extracts influence the cell surface attachment. This was done according to a previous method [20] with some modifications. The effect of the extract on

viral attachment showed significance reduction ($p < 0.05$). Here, the control showed (31 $\log_{10}$) PFU/ml, (12 $\log_{10}$) (HS-treated) and (9 $\log_{10}$) PFU/ml (ZA-treated) (**Figure 5**). This implies that there were significant viral reductions ($p < 0.05$) among or between the two extracts. It also reveals that ZA has more effect on disruption of effective viral attachment when compared to HS.

The results revealed that the three experiments were potentially active in suppressing plaque formation (**Figure 2**; **Figure 4**; **Figure 6**). Experiment 2 was the most effective result indicating that the extracts have a significant effect on viral replication with ZA showing the better of the two. The influence on viral replication showed control (25 $\log_{10}$) PFU as against the HS treated-virus-infected cells (9 $\log_{10}$) PFU/ml and ZA-treated-virus-infected cells (5 $\log_{10}$) PFU/ml.

5. Discussion

Treatment of infectious diseases has been of great interest to health care providers and researchers mainly as the continuous problem of drug-resistant strains has been on the rise globally [21]. Plants naturally contain bioactive compounds (phytochemicals) that protect them from insect and microbe attacks [11]. These phytochemicals have been found effective against bacterial infections by several mechanisms which include inhibition of the activity of toxins and enzymes destruction of virulence factors and damage to the bacterial membrane [12]. This study aims to elucidate whether plaque reduction is due to an effect of the extract directly on the virus, or whether it affects viral replication, whether the extract impact was on the cell surface attachment with the virus.

We determined different polyphenol-enriched extract treatment effects on murine norovirus. These extracts were derived from *Hibiscus sabdariffa* and *Zanthoxylum armatum* whose phytochemical profiles were studied. We demonstrated for the first time that HS and ZA disrupt murine norovirus from easy and useful attachment to the cells (**Figure 6**), unquenchable viral replication (**Figure 4**), and viral plaque reduction (**Figure 2**). **Figure 6** shows the most effective condition; the extract was able to disrupt further viral replication in the cells. Hence, it arrests the possibility of establishing infection. One of the most acceptable methods of quantifying infectious viruses is plaque assay. A viral plaque is a visible arrangement formed within a cell culture. Effective plaque formation is mostly shaped by many factors: virus growth conditions, the health of the cell line, host strain, strict protocol procedure, and culture reagents. Our results have shown that each of the above steps has an impact on viral plaque reduction. The results show that the plant extracts have demonstrated diminished viral development which is evident with less plaque formation in the extract-treated cells. The importance of this result will assist in preventing/controlling norovirus infection. Currently, there is no effective human norovirus treatment. Studies have demonstrated that plant extracts have shown damage to the bacterial membrane [12]. Saponins from *Zanthoxylum armatum* were effective against human breast cancer cells (MCF-7, MDA-MB-468) and

colorectal cancer cells (Caco-2) [14], antiviral [16]. Meanwhile, the impact of the extract on viral replication (step 2) showed a more significant viral reduction at level ($p < 0.05$), when compared to impact directly on viruses and viral attachment]. Inhibition of viral replication is achieved by using an antiviral drug which interferes with viral biosynthesis [22]. Studies have also shown that natural products act as inhibitors of prostaglandin E2 and pro-inflammatory 5-lipoxygenase-derived lipid mediator biosynthesis [14]. Our study has demonstrated *that Zanthoxylum armatum* shows a better inhibitory effect (5 $\log_{10}$) PFU/ml when compared with *Hibiscus sabdariffa* (9 $\log_{10}$) PFU/ml and the control (25 $\log_{10}$) PFU/ml (**Figure 3**). This simply means that the studied extracts have a significant effect on impeding viral multiplication. Viral replication involves chemical recognition and attachment to the appropriate host cell, the whole virus or its genetic material alone enters the cell's cytoplasm, the information contained in the viral DNA/RNA directs the replication of viral nucleic acids, capsid protein, and synthesis of viral enzymes which are packaged as new viral particles and eventually released [23]. This whole process of viral replication has been shown to be hindered with the studied extracts; *Zanthoxylum armatum* and *Hibiscus sabdariffa.* The extracts acted on the virus to such an extent that replication was reduced. *Zanthoxylum armatum* has consistently shown more than 4-log scale reductions in all three experiments although extracts of *Hibiscus sabdariffa* also showed more than 4-log reduction in experiments 2 and 3. For example in experiment 2, *Hibiscus sabdariffa* had up to 16 logarithmic viral reductions while *Zanthoxylum armatum* had 18 logarithmic reductions. Plants are made up of various categories of phytochemicals that prevent them from natural threats (viruses, fungi, and bacteria). Our study has shown that both extracts can reduce the ongoing virus replication, and reduce viral attachments to the host cells [24]. We have demonstrated that *Zanthoxylum armatum* has reduced viral replication on more than a 4-log scale [25]. Studies have also shown that extracts of *Rosmarinus officinalis* plant extract have an inhibitory effect on *S. aureus*, *E. coli*., and *P. aeruginosa* [26]. Our experiment number 3 has also shown a similar result of 14 $\log_{10}$ PFU/ml and 10 $\log_{10}$ PFU/ml for HS and ZA respectively pointing to the effect of biochemical compounds present in the studied plants. In similar studies more than a 4-log scale impact is a promising sign of a strong anti-viral effect [23]. *Zanthoxylum armatum* and *Hibiscus sabdariffa* have demonstrated their potential to lessen both viral replication and modifications of viral attachment.

Phenolic compounds of HS have been reported to possess inhibitory effects on the herpes simplex virus (HSV) [27]. HS has also shown an antibacterial effect against several bacterial strains example *Staphylococcus aureus*, and *Clostridium sporogenes* [28]. This shows that *Zanthoxylum armatum* and *Hibiscus sabdariffa* extracts could be used for the therapeutic development of norovirus. The methanol extract of Hibiscus sabdariffa was inhibitory to *E. coli* O157:H7 [25]. The respiratory syncytial virus, a single-stranded RNA virus has been shown to be

hindered from infection by small therapeutic molecules that bind the glycoprotein and inhibit membrane fusion [29]. Studies have shown that flavonoids (Gossypetin and Taxifolin) obtained from Hibiscus sabdariffa have shown better binding energies in ebolavirus receptors and are currently used on humans to treat Ebola infections [30]. It is noteworthy to remember that the Ebola virus is a negative-sense, single-stranded RNA virus that causes severe hemorrhagic fever in both non-human primates and humans [31]. *Zanthoxylum armatum* aqueous leaves extract has shown antidiabetic properties in both *in vitro* and *in vivo* studies [32]. Studies have shown that methanol and aqueous extract of dried fruit *Zanthoxylum armatum* showed inhibition of HSV-1, influenza, and Japanese B encephalitis, anti-bacterial against *Staphylococcus aureus* and *Bacillus subtillis*, *E. coli*, and *Salmonella typhi* [33].

6. Conclusion

Our study has shown that a 10% concentration of *Hibiscus sabdariffa* and *Zanthoxylum armatum* disrupt murine norovirus from the useful connection by weakening virus attachment and disrupting consistent viral replication. Our results have demonstrated that both *Hibiscus sabdariffa* and *Zanthoxylum* could potentially control norovirus infection. We recommend further studies with animal models. The use of animal models will assist in obtaining more information on diagnosis and treatment.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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