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Electron Micrographic Representations of Mechanisms of Action of Murine Norovirus on ATCC TIB-71 Cells and Level of Gene Expression

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Abstract

Human noroviruses (HuNoV) are the number one cause of viral gastroenteritis worldwide resulting in a significant cause of morbidity and mortality in all age groups. However, despite the medical relevance of HuNoV, effective treatment against norovirus infection is yet to be developed. In this study, we investigated the anti-Noroviral activity of *Hibiscus sabdariffa* (HS) calyces and *Zanthoxylum armatum* (ZA) seeds using murine norovirus, a surrogate of human norovirus. The antiviral mechanisms of action were also examined using a gene expression studies and transmission electron microscopy. Our results showed that virus-infected cells were left potentially void of all the cell machineries whereas uninfected cells represent healthy normal and dividing cells. The infected treated cells with extracts showed restoration of the dense cytoplasm, cytoplasmic membrane, and the nucleus. These cells were also associated with the expression of ORF genes. This study demonstrates the antiviral properties of *Hibiscus sabdariffa* (HS) calyces and *Zanthoxylum armatum* (ZA) and thus indicates their potential as natural remedies to treat noroviruses.

Keywords

Norovirus, Plant Extracts, Transmission Electron Microscope, Prevention/Control

1. Introduction

Norovirus outbreaks are rampant in the nursing homes, military, and cruise ships. The HuNoV is sporadic [1] and occurs mostly in winter [2]. The virus causes about 75% - 90% of nonbacterial gastroenteritis [3] [4], with vomiting and diarrhea as its earliest symptoms [5]. Approximately 685 million cases occur annually worldwide, and approximately 21 million cases occur every year in the United States alone [6]. Nearly 71,000 hospitalizations, 800 deaths, and $493 million in economic losses are accrued because of norovirus infection per year in the United States [3] [7].

The management of this viral infection is presently only through practices such as confinement of infected persons, constant handwashing, and drinking a lot of fluid [8]. There is a dire need for more permanent treatment options as well as their mechanism of actions. Plants are natural resources that have been proved to be effective against viruses with little or no side effect [9]. Therefore, developing resistance against plants is unlikely [10]. Plants contain phytochemicals that naturally protect them from microbe and insect attacks [11]. These phytochemicals carry out their antimicrobial activity through various drug targets, for example, destruction of virulence factors, inhibition of toxin and enzyme activities, and bacterial membrane [12]. Studies have demonstrated that *Warbuga ugandensis plectrunthus barbatus*, *Withania somnifera*, and *Prunus africana* exhibited fungicidal, bactericidal, and immunopotentiation as likely mechanisms of action [13]. Another studies confirmed mechanisms of action of plants by leakage of cellular contents and permeabilization of the inner membrane [14] and also that *Polygonum chinense* is a multi-targeted inhibitor of influenza A and B [15]. The use of herbal plants to treat human diseases has opened an alternative door for treating some of the diseases that are eluding orthodox medicine. The surge in traditional plant use to find permanent solutions to ever emerging humans' disease may provide the long-awaited answer to handling human noroviruses. The curative potential of herbal plants has been sought by scientists over a couple of years. These plants have proven to possess anti-inflammatory, antiviral, antitumor, antimalarial and analgesic properties [16].

Our objective of this study was to determine the mechanism of action through which the phytochemicals modulate virus infection. Bioactive compounds such as polyphenols, flavonoids, and organic acids are known to have therapeutic potentials for most human ailments. Flavonoids block prostaglandin synthesis, cell cycle progression, and protect the cell against injury caused by X-rays [17]. Polyphenol and organic acids are known to regulate enzyme activities and proliferation of bacteria, respectively [18]. The plants of interest in this study are *Hibiscus sabdariffa* and *Zanthoxylum armatum*, which are known to possess flavonoids, polyphenols, and organic acids. Flavonoids like l-epicatechin, tangeretin, and naringenin showed antiviral effect against feline calicivirus (FCV-F9) [19].

Negative staining, a conventional method used in diagnostic microscopy for contrasting a thin specimen with an optically opaque fluid, is an essential technique in electron microscopy [18] [20]. Negative staining requires acidic dye such as uranyl acetate; this means that the stain gives up a proton (a hydrogen ion) and the chromophore of the dye becomes negatively charged [21]. The background is stained, leaving the actual specimen untouched [22] [23]. The virus cell surface repels the stain because the surface of most viral cells is negatively charged. The virus will show up as thin spots against a dark background [23].

2. Materials and Methods

Specimen processing for transmission electron microscopy (TEM): Both treated and untreated RAW 264.7 cells (controls) were fixed in 4% buffered glutaraldehyde and kept in the refrigerator until use. Treated means that RAW 264.7 cells were initially exposed to murine norovirus for few hours and then treated separately with extracts of *H. sabdariffa* and *Z. armatum*. The control groups were normal cells without virus exposure and cells with virus exposure respectively. Glutaraldehyde is incomparable in cell structure preservation [18]. The glutaraldehyde was aspirated and carefully washed with 0.1 M sodium cacodylate buffer containing 7.5% sucrose, three changes for 15 minutes each. Sucrose increases the osmolality of the sample. The samples were post-fixed in 1.0% osmium (OsO_4) in 0.10 M sodium cacodylate buffer for 1 hour on the rotator, washed in 3 changes of 0.11 M veronal acetate (VA) working buffer, 15 minutes each change. The samples were into *en bloc* stain (**Figure 1(A)**) (0.5% uranyl acetate in veronal acetate buffer) for 1 hour and dehydrated in a series of ascending acetone concentration [70%, 95%, 100% (X^2)] for at least 10 minutes each. Furthermore, the samples were placed in a 50/50 mixture of acetone and 100% epoxy resin for at least 1 hour on a rotator. The 50/50 mixture was replaced with 100% epoxy resin for 30 minutes at room temperature on a rotator. The second change of 100% epoxy was made and incubated for another 30 minutes at room temperature on a rotator. The samples were placed into molds with a label and baked for 24 hours for embedding. Sections of grid were cut

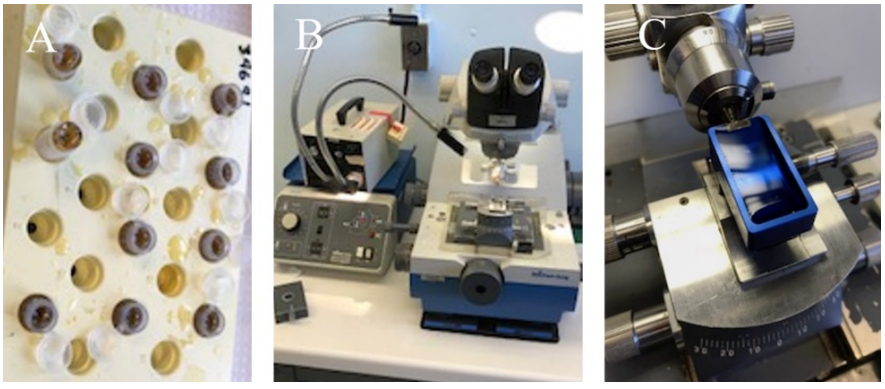

Figure 1. Thin-section process. Samples in bloc (A), ultra-microtome ((B) and (C)), Sections are cut under water (C).

with a diamond knife on an ultramicrotome (**Figure 1(B)** and **Figure 1(C)**) to produce thin slices of samples that were semitransparent to electrons. The sections were about 70 nm thick and stained with uranyl acetate. Uranyl acetate is a heavy metal that gives quality contrast to the image as electrons are scattered for good eye contrast. The grids were air-dried and carefully loaded into the electron microscope (EM) (Phillips CM12) for view while the room was kept dark.

Northern blotting: We employed the northern blotting technique to measure the amount and size of RNA in both treated and untreated samples [24]. We employed a previously described method [24] with modifications. Briefly, samples were run on a denaturing gel to separate RNA according to their sizes (28 S & 18 S). The agarose gel (Invitrogen, Lot no 00607575) was run at 75 V for 55 mins and then moved to a positively charged Biodyne B Pre-Cut modified nylon membrane (0.45 μM, Thermo Scientific Rockford, IL) for 2 hours using NorthernMax-Gly kit (Thermofisher, AM1946).

The labeled probe corresponding to the gene of interest was generated with RT-qPCR as well with traditional PCR products. We used Fotodyne (Incorporated Foto/Analyst luminary FX with darkroom controls) to determine probe efficiency. We hybridized the generated probe to the nylon membrane with transferred RNA in the nylon membrane. Unhybridized probes were removed by washing in several changes of buffer. The solid nylon membrane with probe precisely bound to RNA of interest subjected to analysis (data not shown).

The reverse transcriptase of polymerase chain reaction of ORF 1, ORF 2, and ORF 3 Genes: ORF (1, 2, 3) levels of gene ID 4246735-7 (A-4) were determined using quantitative reverse-transcriptase real-time polymerase chain reaction (RT-qPCR). ORF (Open Reading Frame) is a norovirus gene which is of three parts: 1, 2, and 3. Each of them encodes different proteins. ORF1 has the specific function of encoding polypeptide with regions of close relationship to cysteine proteinase, helicase, and RNA-dependent RNA polymerase (RdRp)-encoding regions. ORF2 encodes viral capsid protein (VP1) while ORF3 encodes small structural protein (VP2) linked with VP1 strength.

Briefly, total RNA was isolated from control and experimental cells using the isolation RNeasy Mini Kit (50) (Qiagen Inc. catalog number 74104), according to the manufacturer's instruction. The total RNA 2 μg was then reverse transcribed using SuperScript-III enzyme (Invitrogen, New York, USA), a 2 pmol of gene specific primer. A non-viral tag sequence attached at the 5' end of the strand specific viral sequence was part of the reverse transcription primer (RT-primer) (A-8). The RT-qPCR targeted the ORFs (1, 2, and 3) genes using gene-specific primers (2 pmol) from Integrated DNA Technologies (IDT) (Ref. no. 187223698). The resulting cDNAs were subjected to RT-qPCR using the MESA Blue qPCR Master Mix Plus for Syber Assay (Eurogentech, Seraing, Belgium). We used a previously described method for the RT-qPCR [25]. Briefly, 2× Mesa blue master mix and the respective primers were mixed with 2 μl of diluted cDNA (A-7). Each strand was at a final concentration of 125 nM before enzyme activation by incubation at 95˚C for 10 min. The cycling parameters in

an Eppendorf realplex^2 (Mastercycler ep gradient S) real time PCR were as follows: 50 cycles of 94˚C, 15 secs; 58˚C, 20 secs; 72˚C, 20 sec. Each sample and a standard curve were run in triplicate to ensure reproducibility.

The ORF 1-3 primers obtained from IDT, Inc. (A-8). ORF 1, 2, 3 PCR primers (TposGpos: 3'-CGGGAAGGCGACTGGAGTGCCCAAACATCTTTCCCTTGTTC-5), qPCR-Tpos Forward: 3'-CGGGAAGGCGACTGGAGTGCC-5', qPCR-RGneg Reverse: 3'-TGGACAACGTGGTGAAGGAT-5') and TnegGneg 3'-GGCCGTCATGGTGGCGCGAATAATGGACAACGTGGTGAAGGAT-5', and qPCR-FTneg GGCCGTCATGGTGGCGAATAA, qPCR-RGpos 3'-CAAACATCTTTCCCTTGTTC-5' utilized were custom synthesized. The $\log_{10}$-transcribed RNA genomic replicas were plotted against the threshold cycle (Ct) value. The positive amplification control and negative amplification control (*Escherichia coli*) were incorporated in each RT-qPCR run.

Statistical Analysis

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

3. Result

To evaluate the mechanisms of action of the virus, we examined virus-infected RAW 264.7 cells by negative staining [18] and thin sectioning. Thin sectioning is a diagnostic procedure, which enhances beam penetration in tissues and cells. Cells were centrifuged and fixed with glutaraldehyde to hold them together. Our result showed that virus-infected cells were left potentially void of all the cell machinery (**Figure 2(A1)** and **Figure 2(A2)**), whereas uninfected cells depict healthy normal and dividing cells (**Figure 2(B1)** and **Figure 2(B2)**). Organelles like the mitochondria, endoplasmic reticulum, cytoplasmic membrane were evident in **Figure 2(B1)** and **Figure 2(B2)**. Treated cells with extracts showed a noticeable difference from the untreated virus-infected cells. The changes in the treated depicted the gentle restoration of some of the virus eroded cell organelles. Typical is the presence of cytoplasmic membrane, dense cytoplasm, and the nucleus (**Figure 3**). In **Figure 4**, we showed the presence and the devastative impact of the murine norovirus in the cytoplasm. Note that this figure consists of untreated virus-infected cells. Murine norovirus is an RNA non-enveloped virus. Our RT-qPCR result shows levels of ORF (1, 2 & 3) gene expression in both treated and untreated cells (**Figure 5**).

To show effects of the extracts *Hibiscus sabdariffa* (HS) calyces and *Zanthoxylum armatum* (ZA) seeds on expression of ORF genes during norovirus infection, active doses of the *Hibiscus sabdariffa* (HS) calyces and *Zanthoxylum armatum* (ZA) seed extracts were added to murine norovirus, a surrogate of human norovirus. Subsequently, the cells were infected with norovirus for 2 hours. mRNA expression levels for ORF family were investigated by utilizing RT-qPCR

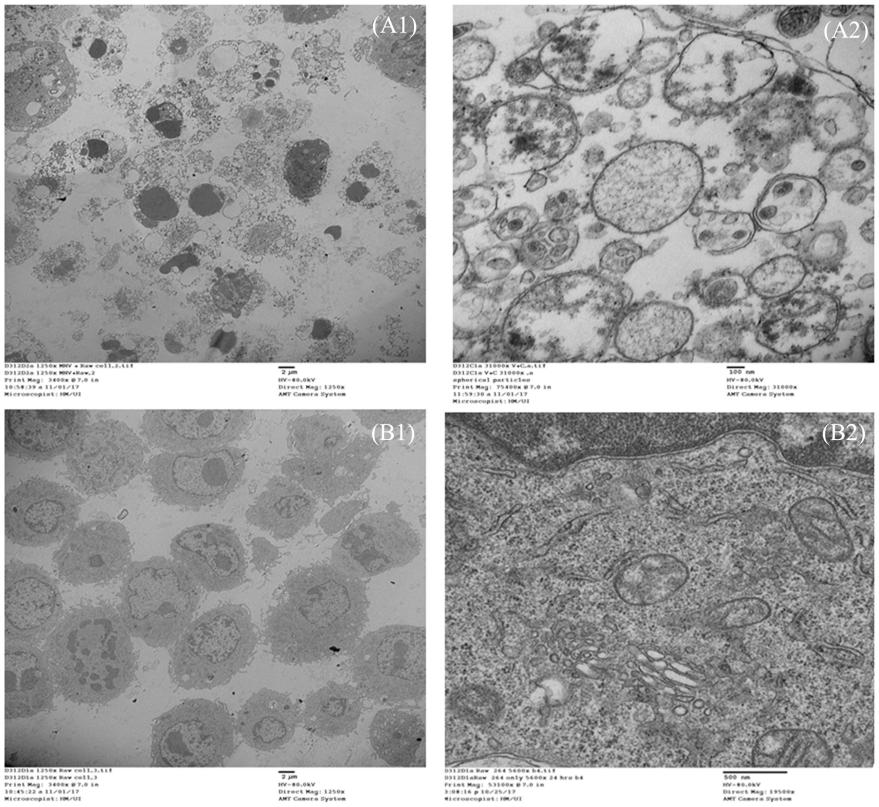

Figure 2. Electron micrographs of untreated samples. MNV+ RAW264.7 ((A1) and (A2)); RAW 264.7 cell line only ((B1) and (B2)).

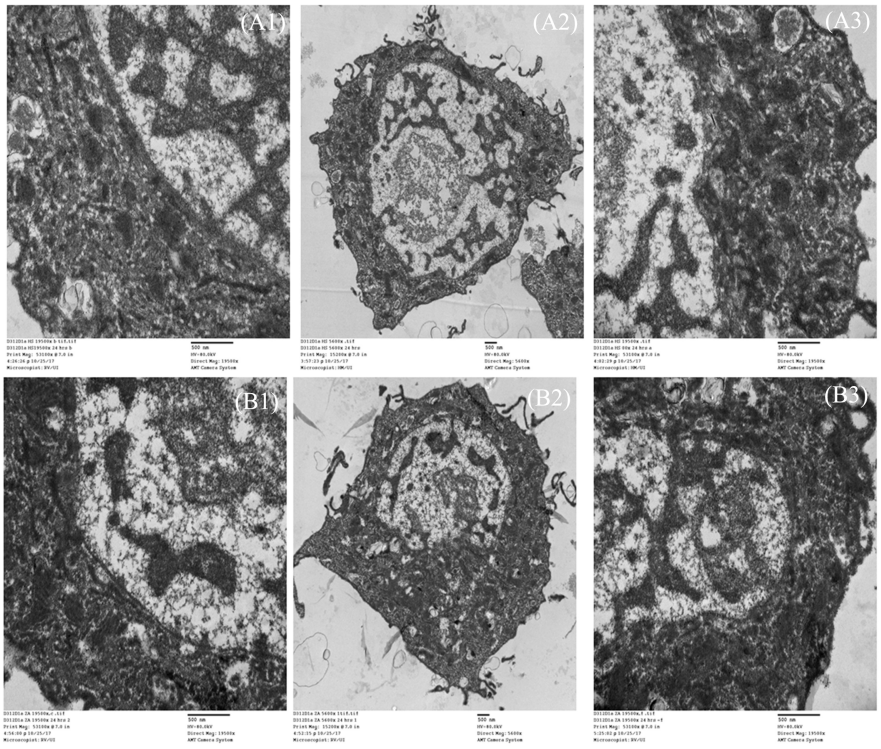

Figure 3. EM micrographs of treated infected cells. Treated with *Hibiscus sabdariffa* extracts ((A1) - (A3)) and extracts of *Zanthoxylum armatum* ((B1) - (B3)).

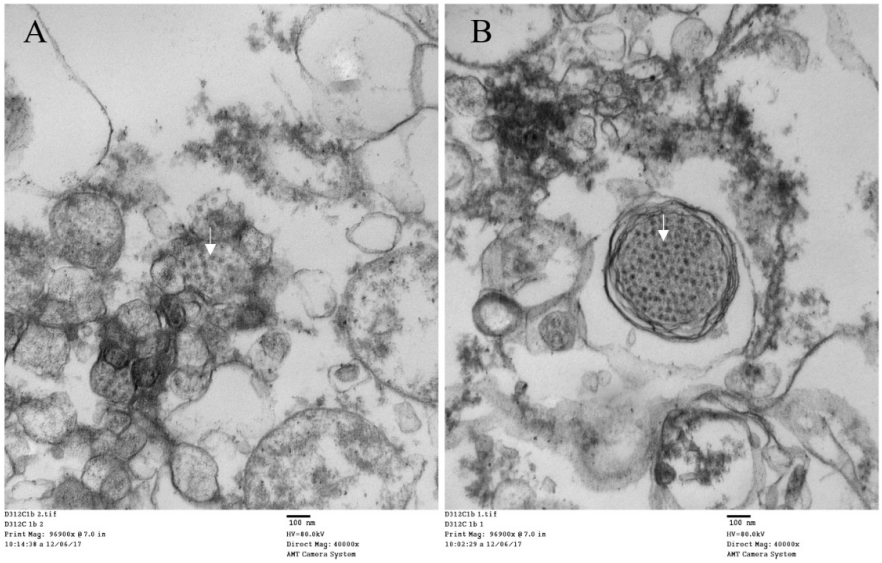

Figure 4. Images of murine norovirus in the cytoplasm. Arrow shows murine norovirus.

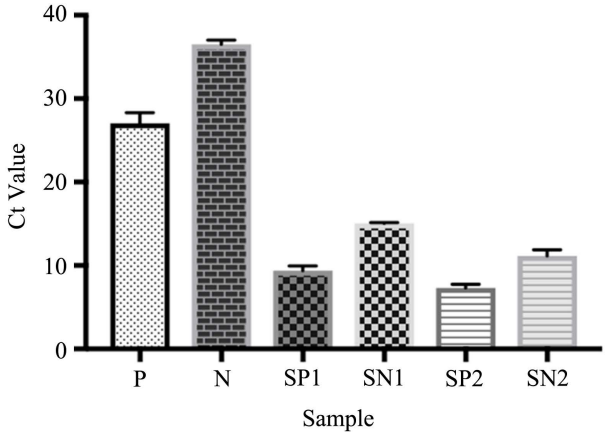

Figure 5. qPCR representation of treated and untreated samples against MNV. P: Positive control, N: Negative, SP1 & SN1: Treated with Hs (+ve & −ve), SP2: Treated with Za (+ve & −ve).

using the MESA Blue qPCR Master Mix. Uninfected cells were used as a negative control. The infected but not plant extract exposed cells were used as positive control. Based on the ORF genes expression analysis results, the expression levels were mainly up (**Figure 5**). The ORF affected by extracts were *Hibiscus sabdariffa* (HS) calyces and *Zanthoxylum armatum* (ZA) seed extracts. Treatment with *Hibiscus sabdariffa* (*HS*) *calyces and Zanthoxylum armatum* (ZA) seed extracts during norovirus infection resulted in the upregulation or expression of ORF as indicated by the Ct values. The Ct values associated with the treated cells (Sp1 and Sp2) were significantly ($p < 0.05$) lower the Ct associated with the control cells (Sn1 and Sn2) (**Figure 5**). The number of expressed gene is inversely proportional to Ct values. The Ct value is inversely proportional to the amount of viral nucleic acid in specimens (*i.e.*, lower Ct values indicate higher amount of virus), so it can be used as a proxy of viral load.

4. Discussion

Plants naturally contain a wide range of secondary metabolites (phytochemicals) which indeed protect them from microbial attack [26] [27]. Phytochemicals are also known to treat an array of human ailments [28] such as cardiovascular diseases [29], diabetes mellitus [30], gastrointestinal tract infection [31], obesity [32], and periodontal diseases [33]. The mechanism through which the studied plants destroy or affect the viral unit is not yet known. In this study, murine norovirus acts as a model to human norovirus to evaluate the inhibitory effects of crude extracts of *Zanthoxylum armatum* and *Hibiscus sabdariffa*.

The plants of interest in this study, *Hibiscus sabdariffa* and *Zanthoxylum armatum*. *H. sabdariffa*, have been widely used as herbal medicine and drinks, food, hot and cold beverages, and as a flavoring agent [34]. The extract has evidently proven to possess antioxidant, neuro-protective [35], antidiabetic, anti-hypertensive [36], anti-cholesterol effects [37]. *Zanthoxylum armatum* has been shown to be anthelminthic, stomachic, and carminative [38]. Both the fruits and seeds control dyspepsia and fever [39].

The objective of this study, therefore, is to determine the mechanism of action of calyces of *Hibiscus sabdariffa* and seeds of *Zanthoxylum armatum* extracts on murine norovirus. Transmission electron microscopy (TEM) was used to observe any structural changes to the extract-treated-cell-murine norovirus complex directly. Transmission electron microscopy (TEM) has aided many virus discoveries—evaluations of virus-host cell interactions as well as diagnosis of various viral infections [40]. TEM has long been used to discover and describe the structure of viruses [41]. The use of TEM has also revealed to have an insight into different virus-cell interactions [42]. In our study, with the help of TEM, we observed the extract-treated-cell-murine norovirus complex directly. We were able to see restoration of cytoplasmic membrane, dense cytoplasm, and the nucleus in the treated cells and the control showing grievously eroded by noroviruses (**Figure 3**) suggesting the extracts acted by arresting viral replication.

Negative staining [18] and thin sectioning were both used in our study. Negative staining is a quick technique used for viewing tiny particles in fluids, typically viruses [43] [44]. Untreated virus-infected-RAW 264.7 exhibited a higher infection rate characterized by the dead cells. Also, the mitochondria cytoplasm, endoplasmic reticulum, ribosome, and nuclei lost their structural integrity (**Figure 2** upper). In contrast, uninfected cells were healthy (**Figure 2** lower). Loss of normal cell integrity (organelles) possibly suggests that the cells may have lost normal physiological processes. Our result agrees with a study of Usutu virus on human neural cells. The study shows that non-treated cells show higher viral infection rate and more cell death [45].

There was evidence of clathrin-mediated endocytosis, a process through which cells absorb proteins, viruses, hormones, and metabolites [46] [47]. Nuclear proteins imported into the nucleus through the nuclear pore complexes (NPCs) [48]. NPCs are large molecular machines that attach to the nuclear envelope

[49]. Stages of cell divisions were seen in this group, chromatins which package RNA were beginning to stick together in the healthy cells. The chromatin aids cell's mitosis and avoids chromosome rupture [50] and hence sustains cell RNA integrity and controls RNA replication and subsequently gene expression [51]. Moreover, the extract treated virus-infected cells showed evidence of change in ultrastructure. The extract acted as a therapy to the infected cells. Our result showed that the extract ordered the cell's machinery in a way that virus could not further replicate. There was evidence of dense cytoplasm, the distinct nuclear membrane separating the nucleus from the cytoplasm. This simply implies that these extracts could potentially be used to treat or control norovirus infection.

Studies have shown that plant extracts have gained a science-based foundation in the treatment of bacterial diseases [52]. For example, the alcoholic extracts of *Hemidesmus indicus*, *Leucas aspera*, and *Tridax procumbens* have shown to involve blebbing and leakage of cellular contents and disruption of membrane potential [14] Antifungal activity of *Momordica charantia* seed extracts is known to cause a loss of integrity of cell wall, disruption of the cell membrane, and deformation of cells with irregular budding [53]. A study of the antiviral effect of *Brazillian Cerrado* against the avian metapneumovirus suggested that the extract acted during the adsorption phase as 99% viral replication was inhibited [54].

Z. armatum has a wide medicinal use. Both the fruits and seeds are typically used in controlling fever and dyspepsia, anthelmintic, stomachic, and carminative, asthma, bronchitis. It has been scientifically proven to be anti-oxidative, anti-inflammatory, antimicrobial, anti-tumor, hepatic-protective, piscidal, insecticidal and larvicidal activities [55].

H. sabdariffa is widely used as herbal medicine and drinks, food, hot and cold beverages, as flavoring agent. *H. sabdariffa* is known to treat various cardiovascular risk factors including hypertension, hypotension, hyperlipidemia in Jordan, Greece, Brazil, and obesity [56] [57] [58]. The extract of *H. sabdariffa* has evidently proven to possess antioxidant, hepatic- and nephroprotective, antidiabetic, anti-hypertensive, anti-cholesterol effects. Anti-toxicity effect of this plant has been proven to be culprit for these multifactorial jobs [59].

The Real-time quantitative polymerase chain reaction (RT-qPCR) is the standard method for gene expression analysis. It is a convenient and fast method that gives the user the opportunity to monitor the PCR reaction in real time [60]. Gene expression is inversely proportional to Ct values (threshold cycle) [61]. The threshold is a point where threshold line intersects reaction curve and a level above background fluorescence usually located at the beginning of the exponential phase. Our study has shown that the positive control had a Ct value of 28 whereas the negative control has a Ct value of 38. This suggests more expression of the gene of interest. We found that higher gene expression of ORF was significantly associated cells treated were *Hibiscus sabdariffa* (HS) calyces *and Zanthoxylum armatum* (ZA) seed extracts. The expression of ORF genes could potentially code for proteins thus providing evidence or support the theory of the

role of restoration of cells organelles in response to plants extracts from the effect of norovirus infection.

It has been shown that Ct values below 29 cycles indicate much availability of targeted nucleic acid and Ct values above 38 cycles show very small amounts of targeted nucleic acids [62]. Our treated samples showed very low Ct values of 10 and 8 (HS and ZA positive) and 15 and 12 (HS and ZA negative), respectively. These values suggest that the amount of gene expression in ZA is insignificantly higher than that those in HS. The genome of interest embodies three open reading frames (ORFs), the gene. The ORF1 has the specific function of encoding polypeptide with regions of close relationship to cysteine proteinase, helicase, and RNA-dependent RNA polymerase (RdRp)-encoding regions. The second ORF (ORF2) encodes viral capsid protein (VP1), and ORF3 encodes small structural protein (VP2) linked with VP1 strength [63]. Treated HS and ZA have shown approximately three-fold and four-fold changes respectively. The fold change reflects the impact of the extracts on the ORF 1 and 2 genes. This further strengthened the activities of macrophages to maintaining structural integrity of the cell. Murine norovirus acts in different ways to the cells including eroding the cytoplasmic contents and invariably affecting the intact nucleus. These norovirus influences invariably cause changes in gene expression of cells treated with plant extracts [64]. In another study, doxorubicin involved in enzyme inhibition and DNA damage subsequently cause changes in gene expression of treated cells [65]. Viral replication depends on the metabolic pathways of the host cell. Many viruses have also evolved to modify many host pathways [66]. Studies have shown that the MNV genome shows a high degree of structural resemblance to that of human norovirus, with the three essential ORFs encoded inside the human norovirus genome having a straight homolog in MNV (A-4) [67]. The murine norovirus genes of interest are three overlapping open reading frames (ORFs). The ORFs encode cysteine proteinase, helicase (ORF-1), viral capsid (ORF2), and minor structural protein connected with VP1 firmness [68].

5. Conclusion

Current advances in molecular diagnostics have helped to establish norovirus as the most common cause of outbreaks of acute gastroenteritis across all ages. *Hibiscus sabdariffa* (HS) calyces and *Zanthoxylum armatum* (ZA) exhibit antiviral activities. Cells treated with HS and ZA extracts were associated with dense cytoplasm, the distinct nuclear membrane separating the nucleus from the cytoplasm from the extract-treated cells which impair replication. Replication was also affected by the expression of several genes.

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Conflicts of Interest

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

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