THE EFFECT OF *MORINGA OLEIFERA* ON THE ONCOLYTIC ACTIVITY OF VESICULAR STOMATITIS VIRUS IN CERVICAL CANCER CELLS

A Thesis
by
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Abstract

THE EFFECT OF *MORINGA OLEIFERA* ON THE ONCOLYTIC ACTIVITY OF VESICULAR STOMATITIS VIRUS IN CERVICAL CANCER CELLS

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Many of the traditional treatment methods for cervical cancer – surgery, radiation therapy, and chemotherapy – are often not cancer-specific and are associated with adverse side effects. Our lab focuses on developing vesicular stomatitis virus (VSV) as an oncolytic, or cancer-killing, agent due to the natural ability of this virus to target susceptible cancer cells. However, some cancer cells have demonstrated increased resistance to infection by VSV. Therefore, we are interested in augmenting the oncolytic activity of VSV by treating cells with natural compounds. Extracts obtained from the plant *Moringa oleifera* have demonstrated anticancer and anti-inflammatory properties. We hypothesize that *M. oleifera* will promote killing of resistant cervical cancer cells by VSV due to their ability to activate immune cells and promote anti-tumor immunity. Of the five extracts of *M. oleifera* examined (aqueous, butanolic, ethanolic, hydroethanolic, and methanolic), the ethanolic extract inhibited the proliferation of C4-II and HeLa cervical cancer cells. This inhibition correlated with decreased levels of NF-κB and Bcl-XL in these cells. In conjunction with

VSV, the ethanolic extract promoted the ability of a wild-type strain of VSV (rwt virus) to kill cells, perhaps by inducing expression of Bax. The methanolic extract promoted killing of SiHa cervical cancer cells by the rM51R-M VSV strain, but did not affect the proliferation of cells. Furthermore, STAT1 levels decreased in VSV-infected cells, suggesting *M. oleifera* may inhibit induction of an antiviral response and promote VSV replication in these cells prior to immune detection. *M. oleifera* stimulated maturation of dendritic cell (DC) subsets and pro-inflammatory cytokines following infection by VSV, indicating the pretreatment with *M. oleifera* has the potential to promote clearance of virus infected cervical cancer cells. Future studies will examine immune cell activation in co-cultures studies and identify active compounds present in *M. oleifera* that promote anti-inflammatory and anti-viral responses.

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Dedication

I would like to dedicate my work and this thesis to my family, who have provided me with continual support. I would also like to dedicate this thesis to my advisor, Dr. Maryam Ahmed, for her unwavering support and much needed advice during the completion of this project.

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Introduction

Cancer is one of the leading causes of death in the United States, with nearly 1.7 million new cases and 600,000 deaths expected to be reported this year alone according to the American Cancer Society Annual Report. Currently approved treatments for cancer include surgery, radiation therapy, and chemotherapy, with more recent and targeted approaches including hormone and immune therapy. The majority of cancer treatments rely on the use of surgery, radiation therapy, and chemotherapy, with individualized treatments typically encompassing the use of a combined treatment involving two or all three of these options (80). However, these traditional methods are associated with undesirable side effects and therefore necessitate the development of innovative treatments that are not only more effective at targeting tumor cells, but will have limited side effects on non-target tissues (52). Therefore, my research has focused on developing alternative treatments for cancer that offer targeted, site-specific approaches with limited side effects.

One of the modern treatments that is currently being researched and has been approved for use in some countries for specific types of cancers is oncolytic virus therapy. The principle for oncolytic virus therapy is based on the premise that viruses can infect cancer cells over normal, healthy cells due to the natural ability of viruses to target susceptible cells. The treatment is characterized by the use of nonpathogenic viruses to target and kill susceptible cancer cells (37). The method by which oncolytic viruses are able to specifically target cancer cells typically depends on the development and characteristics of

the cancer cells and the virus itself. Killing by oncolytic viruses focuses on a distinguishing mechanism associated with the life cycle of viruses– that is, the nature of viruses to induce apoptosis or cause cell lysis following replication of newly synthesized virions (72). Methods by which oncolytic viruses are able to target cancer cells relate to mutations in cancer cells themselves. A common feature associated with carcinogenic cells is their tendency to acquire mutations in anti-proliferative genes, including genes involved in the type I interferon (IFN) antiviral response (31). The type I IFN response is also the main antiviral response controlling virus replication and spread (72). Therefore, mutations in the type I IFN antiviral pathway render cancer cells sensitive to infection and killing by various viruses. By taking advantage of these mutations, oncolytic viruses have the potential to replicate to a higher degree in some cancer cells in comparison to non-cancerous tissue which are still able to mount an antiviral response following infection (4, 30).

The general mechanism underlying activation of the type I IFN antiviral signaling pathway begins with recognition of viruses by cellular pattern recognition receptors (PRRs) and additional sensors in the cytoplasm of cells. Following recognition, downstream activation and transcription events occur, culminating in the production of various pro-inflammatory cytokines including type I IFNs, such as IFNα and IFNβ (44, 71). IFNβ, which has previously been demonstrated to have autocrine and paracrine effects on infected and neighboring cells, respectively, binds to IFN-associated receptors (IFNAR) on cells (71). This binding event leads to the activation of the type I IFN response pathway, which is also known as the Janus Kinase and Signal Transducer and Activator of Transcription (Jak-STAT) pathway. Jak1 and Tyk2 localize to the IFNAR for phosphorylation and downstream co-localization and phosphorylation of STAT1 and STAT2 (71). The STAT1/2 heterodimer

translocates to the nucleus for transcription of targeted genes to increase production of IFNβ and IFNα (71). In addition to the production of type I IFNs, cells are also altered to have increased protection from subsequent infection by viruses via activation of the host immune response (71). For example, various antiviral genes or interferon-stimulated genes (ISG) are transcribed following interactions with IFN and its associated receptor. Three proteins that are involved in the antiviral response that have been heavily researched include Mx1, 2'-5'-oligoadenylate synthetase 1 (OAS1), and protein kinase R (PKR) (78). Specifically, the Mx protein has been shown to disrupt viral genome expression and new virion construction (42, 25). The OAS1 protein has been observed to degrade viral RNA via activation of ribonuclease or RNAse L enzyme (18, 82). Furthermore, viral RNA detection by PKR typically leads to downstream activation events to ultimately inhibit gene translation in host cells, thus preventing synthesis of viral components (23).

In addition to specific genes that are activated during a viral infection, host immune responses also play a key role in innate and adaptive immunity. Initial detection by professional antigen presenting cells (APC), including macrophages and dendritic cells (DC), leads to expression of viral antigens on major histocompatibility (MHC) receptors on APCs (81). Additionally, natural killer (NK) cells can be activated by different signaling molecules including IFNγ and type I IFNs. NK cells detect cells exhibiting down-regulated MHC receptors, a common feature of virus-infected cells, and initiate cell lysis to eliminate these virus-infected cells (17, 50). Much focus has been placed on the stimulation of DCs and the pivotal role they play in oncolytic virus therapy. DCs can be stimulated by a wide variety of pathogens including viruses. Additionally, there are various subsets of DCs that can interact with each other to promote clearance of virus infected cells. Plasmacytoid DCs (pDC) have

previously been shown to be activated by viruses through detection of the virus by toll-like receptor (TLR)-7 (7, 26). Activation and maturation of pDCs, characterized by the up-regulation of costimulatory molecules and production of pro-inflammatory signals such as IL-6, leads to the secretion of the type I IFNs as well as activation of myeloid DCs (mDC) (7). The myeloid subset of DCs is typically associated with downstream activation of T cells, such as $CD8^+$ cytotoxic T cells (10, 55). Activation of T and B cells is associated with adaptive immunity, characterized by the production of antibodies as well as memory B and T cells to provide heightened protection from future infections (17, 28). Given their pivotal role in viral clearance of infected cells, DCs and other key immune cells may serve as crucial mediators in the removal of virus-infected cancer cells. Therefore, during oncolytic therapies, a desirable response is one that initiates activation of DC subsets to promote anti-tumor immunity. As part of my project, I will be testing whether DC subsets are stimulated following my therapeutic approach.

One virus currently being researched for use in oncolytic therapy is vesicular stomatitis virus (VSV). This virus is a negative-sense, single-stranded RNA virus in the Rhabdoviridae family. While the virus is pathogenic to certain livestock, it is considered nonpathogenic to humans, and its infection is often characterized by mild flu-like symptoms. Its genome encodes only 5 proteins, which allows for easy manipulation. The nucleocapsid (N) protein encloses the RNA material, while the phosphoprotein (P) and large subunit (L) protein function to catalyze replication of the VSV genome with the use of the RNA-dependent RNA polymerase (94). The glycoprotein (G) allows attachment of the virus to the host membrane. The matrix (M) protein is the virulence factor of the virus and is responsible for the pathogenic effects associated with VSV infections (94).

VSV has previously been shown to bind to phosphatidylserine, a phospholipid ubiquitously present on most animal cells, allowing VSV to bind to most cells (59). It is important to note that the specificity of VSV for cancer cells over normal cells is not due to differences in surface receptors, but due to deficiencies in protective mechanisms in cancer cells versus normal cells. While normal, healthy cells are capable of inducing an antiviral response through IFN production as previously described, cancer cells do not respond in a similar manner, as it serves as a disadvantage to their own survival (13, 36). Defects in the IFN response contribute to the susceptibility of cancer cells to infection with VSV (36). In contrast, neighboring, healthy cells are resistant to infection and killing by VSV due to intact antiviral signaling pathways (86). However, as shown previously, as well as described in my research, cancer cells exhibit varying susceptibilities to virus infection (4, 67). Therefore, not all cancer cells have profound defects in the type I IFN pathway. My research seeks to investigate mechanisms to enhance the ability of VSV to infect and kill resistant cancer cells by breaking down their barriers to virus infections.

The ability of VSV to kill cancer cells is credited to the M protein. The pathogenicity of this protein is partly related to its involvement in the budding process. Following the production of newly synthesized virions, each virus particle associates with the host cell membrane to exit the host cell. The M protein initiates and aids in the budding process. The release of thousands of VSV virions from a host cell typically leads to cell lysis (6). Furthermore, the M protein plays a key role in the cytopathology associated with VSV. The M protein suppresses the expression of newly synthesized host genes by inhibiting host transcription, nuclear-cytoplasmic transport of host mRNA, and is involved in the shut-off of host translation. These mechanisms of suppression aid in supporting replication and survival

of the virus within a host (6, 94). Furthermore, the overall suppression of host gene expression leads to the depressed expression of genes in the type I IFN antiviral response (7). While this activity is desirable to effectively kill cancer cells, adverse side effects could potentially occur in healthy cells due to the inability of normal cells to protect themselves against virus replication and killing. Recombinant strains of VSV with mutations in their M proteins are therefore used to decrease the potential pathogenicity in non-cancerous cells and allow for the production of IFN in normal, healthy cells (7). A recombinant strain that has exhibited these characteristics has been previously described by Ahmed et al. (6). The rM51R-M viral strain of VSV, which has an arginine substituted for a methionine at position 51 of the M protein, is defective at inhibiting host gene expression, including expression of genes in the type I IFN antiviral response (7).

While rM51R-M virus has shown much promise to serve as an oncolytic virus, the effectiveness of killing certain cancer cells is further influenced by the characteristics of the cancer cells alone. As mentioned earlier, some cancer cells have been shown to be fairly resistant to infection by VSV due in part to their ability to maintain effective antiviral responses (4). One method that has been used to increase the effectiveness of killing cancer cells with VSV is by inserting different cytotoxic genes into the genome such as thymidine kinase (TK) (33). Fernandez and colleagues show that following infection of melanoma cells with the engineered VSV strain expressing TK, subsequent treatment with the prodrug, Ganciclovir, induced cell death through interactions with DNA replication. In addition, certain strains of VSV have an added gene that will increase the immune response following infection, such as interleukin-4, an important cytokine involved in the stimulation of key immune-modulatory cells (33, 36). Therefore, it is becoming clear that alternative and

combination approaches for oncolytic therapies may exhibit greater efficacy. I am interested in developing combination therapies involving the use of the rM51R-M virus because it serves as a safer option for treating cancer. In addition, previous studies have shown rM51R-M virus is more effective than wild-type strains of VSV at stimulating both mDC and pDC (7). Therefore, this strain has the potential to promote clearance of virus-infected cancer cells by promoting activation of an immune response following stimulation of the IFN response (6).

It is important to appreciate the alterations that arise within individual cancer cells during carcinogenesis, as this will ultimately influence the response exhibited following treatment with oncolytic viruses such as VSV. Initial changes involve mutations to promote increased supply of growth factors and immunity to anti-proliferative signals to promote apoptotic evasion. Other events, including increased blood supply via angiogenesis, are necessary for tissue invasion and metastasis to occur (40, 43). These mutations often occur in pro-oncogenes and tumor suppressor proteins that lead to constitutive activation of key proliferative pathways such as the nuclear factor-kappa B (NF-κB) and Akt pathways (3, 63, 69). Therefore, many treatments focus on these pathways as a means to inhibit the proliferation and survival associated with carcinogenesis. Additionally, these pathways have also been implicated in oncolytic therapy responses, with some research suggesting that activation of these pathways aids in virus replication within cells (79).

The NF-κB pathway is associated with cell survival, serving as an anti-apoptotic transcription factor (58). NF-κB is associated with transcription of more than 100 genes, many of which are associated with regulation of the immune system, but includes other responses to stimuli associated with stress and pathogenic infections (63). While there are

variations to its activation, a generalized activation of the NF-κB pathway is initiated once specific stimuli – such as pro-inflammatory cytokines including IL-6, TNF, and IFNα – bind to their respective receptors on a cell surface. Once bound, the IKK complex is activated and phosphorylates IκB (IκBα). This results in two outcomes: the IκB inhibitory subunit is degraded by the 26S proteasome and the now active p50/p65 subunit (NF-κB) translocates to the nucleus for transcription of target genes (8, 38, 27, 63).

The NF-κB pathway plays a key role in the induction of immune cells. This signaling pathway is involved in the survival and activation of immune cells associated with both the innate and adaptive immune responses including NK cells, neutrophils, B cells, and T cells (45). The NF-κB family has been shown to induce dendritic cells into their matured state, characterized by increased expression of costimulatory molecules for T cell activation in combination with the secretion of other inflammatory cytokines (56, 75).

Tumor cells have also been shown to have increased activation of NF-κB. While constitutive activation of NF-κB is a common feature in proliferating immune cells such as T and B cells, increased levels and constitutive activation of this transcription factor in non-immune cells is associated with tumor promotion. Therefore, in cancer cells, NF-κB acts as an oncogene to influence cell survival and growth (3, 39). In addition to deregulation of specific transcription factors, such as NF-κB, in cancer cells, tumor microenvironments are associated with the presence of pro-inflammatory cytokines such as TNF, IL-23, and IL-6 (39). These cytokines increase expression of genes involved in supporting cell growth, survival, proliferation, and metastasis (63). Therefore, the cancer phenotype and tumor environment develops as a result of a variety of changes in the cancer cells as well as its microenvironment.

The Akt pathway, also referred to as the protein kinase B (PKB) pathway, is another pathway associated with cell survival and anti-apoptotic signaling (46, 64, 92). Activation of the Akt pathway occurs through various signals, which is commonly initiated following the binding of cytokines to their associated receptors. Recruitment of phosphatidylinositol-3 kinases (PI3K) to the cell membrane follows, which can lead to the activation of Akt and downstream transcription of target genes associated with cell proliferation, differentiation, and angiogenesis (48, 92). The Akt pathway is also associated with immune cell activation including dendritic cells, B and T lymphocytes, NK cells, neutrophils, and macrophages (21, 49, 54, 60).

Akt acts as an upstream regulator of various factors associated with apoptotic and anti-apoptotic pathways such as the Bcl-2 family and the caspase family, with support showing that these downstream factors can lead to the induction of cancerous growth (19). Overexpression of Akt genes and mRNA have been found in multiple types of cancer, including breast, ovarian, cervical, and prostate cancer cells (19, 69). Furthermore, overexpression of Akt in malignancies is fairly common, further supporting its role in the development and progression of cancer (69).

Both of these pathways have also been implicated in activation of an antiviral immune response. Following activation of the Akt pathway via binding on viral antigens to TLRs, downstream interactions with interferon regulatory factors (IRF) will activate the type I IFN response for viral clearance. For example, detection of VSV by TLR-4 leads to phosphorylation of Akt, which then activates with IRF3 and the type I IFN response (97). The activation of the NF-κB pathway has been exhibited through interactions with multiple types of viruses (47). While in some cases gene transcription following DNA binding by NF-

κB subunits can actually promote viral replication and survival in infected cells, the NF-κB pathway can also lead to increased protection from apoptosis induced by the lytic nature of viral blebbing (47). Therefore, by determining whether these pathways are activated or inhibited, I will be able to assess their contributions during oncolytic therapies.

Mutations in factors other than NF-kB or Akt are also found in cancer cells to support increased resistance to apoptosis or programmed cell death (43). Alterations in the levels of Bcl-2 family members are commonly found in multiple types of cancers. For example, increased levels of the anti-apoptotic factor, Bcl-XL, have previously been observed in some cancer cells (1, 43). Furthermore, increased levels of Bcl-XL leads to an inhibitory effect on other members of the Bcl-2 family including the pro-apoptotic factor Bax. Located in the mitochondrial membrane, Bax induces apoptosis by releasing various apoptotic factors, such as cytochrome C (1, 43). Therefore, an optimal environment is established that supports survival and proliferation of cancerous cells following inhibition of pro-apoptotic mechanisms.

My research has focused on examining the therapeutic effect of oncolytic VSV on cervical cancer. One of the more prevalent cancers in women worldwide, cervical cancer is still associated with thousands of deaths per year (29). Additionally, greatly than 99.7% of all cervical cancer is associated with infection by the human papillomavirus (HPV) (15). Research has shown that the transition from healthy to cancerous growth does not occur directly following infection by HPV, but is due to the persistent infection of what are categorized as high risk HPV strains within an individual (77). Over 70% of infections by high risk strains that lead to the development of cervical cancer are associated with infection by HPV-16 and HPV-18 strains (29). Of the proteins encoded for by HPV following its

integration in the host cell's genome, most attention has focused on the E6 and E7 proteins and their ability to suppress the functions of the tumor suppressors, retinoblastoma (RB) and p53 (TP53) proteins (29, 77). The RB protein is associated with extracellular signaling to inhibit cell growth and division following detection by various inhibitory growth signals (43). The p53 protein, however, will typically inhibit cell growth and division based on intracellular signaling events that can be associated with stress signals as well as detection of erroneous DNA within a cell (43). Research has shown that the E6 protein binds to the E6-associated protein (E6AP) to target p53 for proteasomic degradation following ubiquination. By doing so, p53 levels are lower in HPV infected cells, supporting cell cycle progression (15, 29). The E7 protein binds to RB, which releases the E2F transcription factor and allows it to translocate to the nucleus to activate genes associated with cell cycle activation and continued division of HPV-infected cells (15, 29). As HPV maintains its integration in host cell genomes and continues to promote cell cycle activation and cell division, mutations arise that ultimately transform a healthy cell into a cancerous cell (77). Given that the E6 and E7 proteins initiate the development of cervical cancer, it is possible that these proteins will influence the responses of cervical cancer to various treatments.

Current treatment options for cervical cancer are dependent on the stage of cancer. Notably, the bulk of treatments for cervical cancer include the use of surgery, radiation therapy, and chemotherapy with most individualized treatments involving the use of a combined therapy of two or all three of these traditional methods (77). Treatment of early-stage cervical cancer is often effective and typically entails minimal tissue removal, but this treatment option can still be associated with post-treatment side-effects such as premature birth (77). Treatments for late-stage cervical cancer often lead to adverse side effects

negatively impacting other organs in an individual. Additionally, late-stage treatments are considered incurable and treatment is focused on prolonging a patient's life expectancy (93).

Newer methods for cervical cancer treatment are focused on more targeted approaches, such as the use of monoclonal antibodies (89). Other targeted treatment options include the use of drugs to target specific growth receptors present on cell surfaces to decrease angiogenesis events associated with sustained cancer cell growth (66). Le Boeuf and colleagues (57) examined the effect of oncolytic VSV on cervical cancer cell killing, and found that the cell lines exhibited variation in sensitivity to infection by VSV strains. Data in our lab has also shown that some cervical cancer cell lines are susceptible to killing by VSV while others are resistant. Some cervical cancer cell lines also demonstrated resistance to killing by different treatment options (53). Therefore, identifying secondary agents that can be used in combination with VSV is one method that may increase cell killing of these treatment-resistant cervical cancer cell types.

The use of VSV in addition to other chemotherapeutic drugs has been examined to increase efficacy of killing resistant cancer cell types. One strategy to increase the effectiveness of VSV in killing cancer cells is through the use of complementary agents such as natural compounds. Previous studies have shown that certain compounds alone and in combination with oncolytic viruses increase cancer cell killing (41). Luo et al. (62) found that the combination of cisplatin and VSV increased cell killing of a murine squamous cell carcinoma line. Studies examining the efficacy of using VSV in combination with natural compounds are sparse, yet a connection between an oncolytic agent and natural and chemotherapeutic compounds is very promising based on the characteristics of both agents.

The goal of this research is to combine oncolytic VSV therapy with *Moringa oleifera* to identify a potential combination approach for the treatment of cervical cancer. *M. oleifera* is a plant native to Asia and Africa, and is cultivated worldwide (68). Parts of the plant, such as leaves, seeds, roots, and flowers, have been shown to have medicinal values (9, 87). For example, the leaves of *M. oleifera* have been shown to be high in various vitamins, amino acids, and minerals associated with anti-inflammatory and anti-cancer qualities (9). Furthermore, other parts of the plant, such as the roots, seeds, and flowers have also demonstrated similar qualities with variation in the compounds present due to the part of the plant used (9). Previous studies have shown that *M. oleifera* aqueous extract inhibits activation of the NF-κB pathway in pancreatic cancer cells. In addition to the involvement of this pathway in cell survival and growth, the NF-κB pathway is also involved in stimulating antiviral responses (16). The solvent used to extract the plant material also influences the chemical composition of the extract acquired from the plant (94). While its use as a medicinal product has been demonstrated for the treatment of various health issues including inflammation, bacterial infection, and cancer alone, its use in combination with oncolytic viruses has not yet been studied (9).

Based on previous studies examining the impact of *M. oleifera* on cancer cell killing, we hypothesize that chemical compounds present in plant extracts from *M. oleifera* will inhibit cell proliferation and promote infection and spread by VSV in cervical cancer cells by altering the activation of certain pro-survival mechanisms or pathways such as NF-κB or Akt. If *M. oleifera* suppresses NF-κB activation in cancer cells, this may allow increased susceptibility to infection and killing by VSV due to the down-regulation of the antiviral response. Furthermore, we have previously shown that VSV also stimulates the activity of

certain immune cells. Therefore, we hypothesize that extracts from *M. oleifera* in combination with VSV will alter the tumor microenvironment by promoting anti-inflammatory cytokines that will suppress cancer cell growth and survival and activate key immune cells such as DCs to promote anti-tumor immunity.

Materials and Methods

Virus strains

The VSV strains used in this study – the wild type rwt strain, the mutant strain rM51R-M, and the GFP expressing strains rwt and rM51R-M were provided from Dr. Douglas Lyles at Wake Forest Baptist Medical Center. The green fluorescent protein (GFP) – expressing strains of VSV, rwt-GFP and rM51R-M-GFP, have the GFP gene inserted into an additional transcriptional unit between the M and G genes of VSV. Therefore, GFP is only expressed during replication of the virus in infected cells. Virus stocks were prepared in BHK cells and kept at -80°C until use. Virus titers were determined using conventional plaque assays. Briefly, BHK cells were grown in 6-well plates and infected with virus stock at different dilutions. At 1 hour, sterile 2X Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% FBS was added in equal parts to 2% agar and added to the cells. Following 24-48 hours of incubation, plates were refrigerated for one hour following the addition of 3.7% formaldehyde to each well. Formaldehyde and agar was removed, and crystal violet was added to each well for visualization of plaque formation and quantitation of viral titers in PFU (plaque forming units)/ml.

Cell Lines

SiHa, C4-II, and HeLa cervical cancer cells were maintained in DMEM supplemented with 10% FBS. All cells were incubated at 37°C in 5% CO_2 (51). All cell lines have shown various degrees of resistance to infection by VSV. Infection by different types of the human papillomavirus (HPV) has been correlated with the development of cervical

cancer. SiHa cells are an immortal grade II, squamous epithelial carcinoma cell line containing human papillomavirus-16 (HPV-16) DNA, and were isolated from a 55 year old Asian female (65, 76). C4-II cells, an immortal squamous epithelial carcinoma cell line containing HPV-18 DNA, were isolated from a 41 year old Caucasian female (11, 96). HeLa cells are squamous epithelial carcinoma cells containing HPV-18 DNA that were the first immortal cell line grown in culture, and were obtained from a 31 year old African American female in 1952 (73).

M. oleifera extracts

To determine potential differences between the solvent used for extraction of *M. oleifera* compounds, 5 leaf extracts were used: (1) methanolic extract, (2) pure aqueous extract, (3) hydroethanolic, (4) pure ethanolic extract, and (5) ethanol to butanol to water (1:1:1). All extracts were provided by Dr. Nathan Mowa of Appalachian State University and Dr. Jahangir Emrani of North Carolina A & T State University. For extraction of compounds, 1000 ml of the designated solvent was used to extract compounds present in the leaves of the plant. Extracted material was then filtered, concentrated, and reconstituted. Each extract was diluted with phosphate buffered saline (PBS), and filter sterilized. The stock extracts were stored at -80°C until needed.

Killing of cancer cells by M. oleifera

To determine the contribution of *M. oleifera* to killing of cervical cancer cell lines, MTT cell proliferation assays (Roche Life Science) were carried out as outlined by Nair and Varalakshmi (68). Briefly, cells were seeded into 96-well plates and grown to 70-80% confluency. Cells were then treated with *M. oleifera* extracts at concentrations ranging from 1 – 200 µg/ml. Following a 6 hour incubation with the extracts, cells were infected with rwt

or rM51R-M strains at a multiplicity of infection (MOI) of 10 plaque forming units per cell (pfu/cell), and allowed to incubate for 24 and 48 hours. A 6 hour pretreatment with the extracts was used to allow for adequate time for potential activation of cellular pathways in response to compounds present in the extracts prior to infection. An MOI of 10 pfu/cell was used to synchronously infect cells at the time of infection. Formazan salts reduced by metabolically active cells were solubilized and absorbance was used to determine the percent of viable cells. Experiments were performed in triplicate for all extracts demonstrating anti-proliferative properties or increased cell killing alone or in combination with VSV following a primary screening.

Effect of M. oleifera on virus replication

To examine the effect of *M. oleifera* on VSV replication, cervical cancer cells were seeded in 24-well plates and allowed to grow to 70-80% confluency. Cell were pretreated for 6 hours with extract concentrations ranging from 0 – 200 µg/ml, at which point cells were infected with rwt-GFP or rM51R-M-GFP VSV strains at an MOI of 10 pfu/cell for 6 hours. Cells were washed with and resuspended in 400 µl sterile, ice-cold phosphate buffered saline (PBS). The percent of GFP-positive cells and the x-mean fluorescence, or the degree of replication within each infected cell, was measured using a Beckman-Coulter Cytometrics FC 500 MPL flow cytometer.

Effect of M. oleifera extracts on cell signaling pathways

To determine the effect of *M. oleifera* on cell signaling pathways associated with proliferation, survival, apoptosis and antiviral responses, Western blot analysis was carried out. Antibodies to phosphorylated and total NF-κB, Akt and STAT1, as well as antibodies to Bcl-XL and Bax were obtained from Cell Signaling Technology. For all experiments,

cervical cancer cells were seeded and grown in 6-well plates to 70-80% confluency and pretreated for 6 hours with 0 – 100 µg/ml of *M. oleifera* extracts. Cells were then infected with rwt or rM51R-M virus at an MOI of 10 pfu/cell for 24 hours. To measure the expression of phosphorylated and total STAT1, cells were treated with 100 IU/ml of Universal Type I IFN (PBL Assay Science) for 24 hours following a 6 hour pretreatment with *M. oleifera* extracts. Plates were then placed on ice at which point supernatants were removed and cells were washed once with sterile, ice-cold PBS. Cell supernatants were collected in RIPA buffer supplemented with aprotinin and stored at -80°C until use. To determine total protein concentration of samples, a Pierce BCA protein assay kit (Thermo Scientific) was used. Samples were electrophoresed using 10% polyacrylamide gels (Bio-Rad) using 15-20 µg of total protein per sample. Following transfer to nitrocellulose membrane, membranes were blocked with 5% dry milk and 0.1% tris-buffered saline-tween (TBS-T) at room temperature (RT) for 1-3 hours. Overnight incubation at 4°C in the primary antibody of interest (Cell Signaling Technology) in 5% BSA plus 0.1% TBS-T was followed by a 1-3 hour RT incubation in goat-anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody. For visualization of signal on Genemate Blue Lite Autorad film, Supersignal (Thermo Scientific) West Pico Chemiluminescent or Dura Extended Duration substrate was used. Quantification of relative band intensities was completed using Quantity One v. 4.6.3 (Bio-Rad). Intracellular concentrations of NF-κB, Akt, Bax, Bcl-XL, and STAT1 were determined and normalized to β-actin.

Effect of M. oleifera extracts on immune cell activation

To identify the impact of *M. oleifera* on immune responses, whole blood was collected from healthy individuals in BD Vacutainer Sodium Heparin tubes. Blood was mixed 1:1 with sterile PBS and layered in a 4:3 ratio on top of Ficoll-Paque PLUS (GE) in conical tubes. Tubes were centrifuged for 35 minutes at 400G at 20-22°C. The layer containing peripheral blood mononuclear cells (PBMC), approximately 3-5 ml, was drawn off using a sterile Pasteur pipette, transferred to a new tube and washed with sterile PBS and centrifuged for 10 minutes at 400G (RT). This step was repeated once, and PBMCs were then resuspended in 5 ml RPMI supplemented with 10% FBS. Cell were counted using a hemacytometer, and suspended at $1x10^6$ cells/ml. 500 ml of suspended PBMCs were transferred into 24-well plates, and immediately pretreated with 0-100 µg/ml of *M. oleifera* extracts for 2 hours, and then either infected with rwt or rM51R-M virus at an MOI of 10 pfu/cell. The effect of the extracts on activation of an immune response was determined by treating cells with 500 ng/ml of lipopolysaccharide (LPS) (Sigma-Aldrich). Following a 20 hour time-point from infection or treatment with LPS, 300 µl of supernatant was collected and stored at -20°C for determining the effect of extracts on pro-inflammatory cytokine production (IL-6 and TNF) by PBMCs using enzyme-linked immunosorbent assays (ELISA) following the manufacturer's protocol (BD OptEIA) and as outlined by Ahmed et al. (5). PBMCs were resuspended in the remainder of supernatant (200 µl), and labeled with fluorescently-labeled antibodies against activation or maturation markers for DC, NK and T cells. DC subset and activation markers included CD11c, CD123 and CD83, while NK and T cell markers included CD56, CD3 and CD69 (Beckman-Coulter).

Cancer cell and PBMC co-culture experiments

To determine the effect of *M. oleifera* on the immune response in the presence of cancer cells, PBMCs were isolated from whole blood as previously described for immune cell activation. Following isolation, PBMCs suspended at $2x10^6$ cells/ml. HeLa cells were trypsinized and resuspended in RPMI supplemented with 10% FBS at $5x10^5$ cells/ml. Equal volumes of HeLa cells and PBMCs were added to a 24-well plate up to a total volume of 500 µl/well. Cells were pretreated for 2 hours with 0-100 µg/ml of *M. oleifera* extracts and subsequently infected with rwt or rM51R-M virus for 24 hours. Supernatant was collected and store at -20°C until use. For controls, HeLa or PBMCs were treated alone, infected alone, or treated and infected alone to determine the effect of the extracts on cytokine levels without co-culture. Cytokine levels were determined with the use of ELISAs following the manufacturer's protocol (BD OptEIA) and as outlined by Ahmed et al. (5).

Results

M. oleifera extracts inhibit cervical cancer cell proliferation and increase cell killing alone and in combination with VSV

Previous studies have demonstrated the ability of *M. oleifera* extracts to inhibit cancer cell proliferation (16, 85). To determine if compounds present in *M. oleifera* inhibit proliferation of cervical cancer cells, the viability of three cervical cancer cell lines, HeLa, C4-II and SiHa, following a 24 and 48 hour incubation with various extracts of *M. oleifera* was determined by MTT assay. The extracts used in this study included the aqueous, butanolic, hydroethanolic, ethanolic, and butanolic extracts, each of which contain distinct compounds that may exert specific effects on these cells. Following treatment of cervical cancer cells with *M. oleifera* extracts (1, 5, 25, 100, or 200 μg/ml), the tetrazole MTT reagent was added to wells to distinguish and quantitate metabolically active cells. Solubilization of these formazan crystals produces a purple coloration which can be measured by spectrophotometry to provide an optical density (OD). Thus, the OD is a measurement of cell viability. To determine if extracts inhibited cell proliferation, cell viability of treated cells at different times post-treatment were compared to the viability for untreated cells at the 0 hour time point. Results indicated that only the ethanolic extract was effective at inhibiting proliferation in cervical cancer cells. As shown in Figure 1A and Figure 1C, respectively, the ethanolic extract significantly inhibited C4-II and HeLa cell proliferation at 24 hours post infection ($p<0.05$), demonstrating an decreasing trend in cell growth compared to the 0 hour mock. At 24h post-treatment, the addition of ethanolic extract as low as 1 μg/ml significantly

inhibited (p=0.048) proliferation of C4-II cells (Figure 1A). HeLa cells were more resistant to the inhibitory effects of the ethanolic extract, such that 200 µg/ml extract was necessary to significantly inhibit proliferation (p=0.019) (Figure 1C). Inhibition of C4-II cell proliferation was also exhibited 48 hours post-treatment, with a significant decrease (p=0.013) in proliferation following treatment with 200 µg/ml of ethanolic extract (Figure 1B). The ethanolic extract did not inhibit HeLa cell proliferation following a 48 hour incubation (data not shown). As mentioned previously, other *M. oleifera* extracts tested did not influence C4-II, HeLa, or SiHa cell proliferation at 24 or 48 hours post-treatment, as illustrated in Figure 1D in which SiHa cells were treated with the methanolic extract for 24 hours.

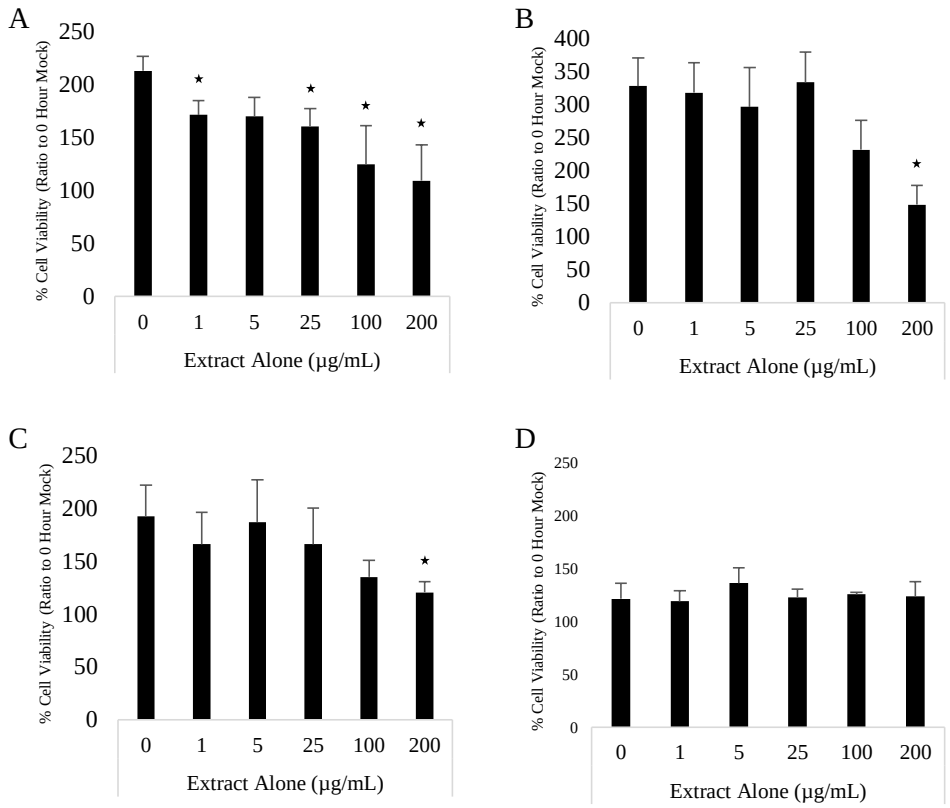

FIG. 1. *M. oleifera* ethanolic extracts inhibits C4-II and HeLa cell proliferation following a 30 hour treatment. Cervical cancer cells were treated for 24 or 48 hours with *M. oleifera* extracts (aqueous, butanolic, ethanolic, hydroethanolic, or methanolic) at various concentrations. C4-II cells were treated for 24 hours (A) and 48 hours (B) with ethanolic extract at concentrations ranging from 0 – 200 µg/ml. (C) HeLa cells were treated for 30 hours with ethanolic extract at concentrations ranging from 0 – 200 µg/ml. (D) SiHa cells were treated with methanolic extract for 24 hours at concentrations ranging from 0 – 200 µg/ml. All five *M. oleifera* extracts were tested on all three cervical cancer cell lines once, and initial experiments showing influence on cell proliferation were repeated twice. Data shown are the mean of three experiments ± standard error. Stars signify significance (p<0.05) as compared to untreated cells.

Previous studies in our lab have demonstrated that natural compounds used in combination with VSV can increase killing of VSV-resistant cancer cell types (data not shown). To determine if *M. oleifera* extracts used alone or in combination with VSV promoted killing of cervical cancer cells, cells were pre-treated with the ethanolic extract of *M. oleifera* for 6 hours and infected with VSV for a total of 24 or 48 hours. Cell viability was determined by MTT assay and data was represented as a percentage of mock infected cells.

Controls included cells treated with *M. oleifera* alone or with VSV alone. *M. oleifera* ethanolic extract alone was shown to significantly promote C4-II cell killing in a concentration dependent manner ($p<0.05$) by 24 hours (Figure 2A). By 48 hours, C4-II cells remained sensitive to killing by the ethanolic extract, but only at the higher concentration of 200 µg/ml ($p=0.012$, Figure 2B). A similar result was seen in HeLa cells following a 24 hour incubation with the ethanolic extract. Results demonstrate significantly lower cell viability ($p<0.05$) at 25, 100, and 200 µg/ml (Figure 3A). An increasing trend in killing of HeLa cells at 48 hours was observed, but was not statistically significant (data not shown). Unlike C4-II and HeLa cells, the ethanolic extract did not alter SiHa cell viability (data not shown). The aqueous, butanolic, hydroethanolic, and methanolic extracts did not affect the viability of each of the cells lines. As illustrated in Figure 3B, SiHa cells remained resistant to the methanolic extract when treated for 24 hours.

Previous studies in our lab have demonstrated that some cervical cancer cell lines (C4-II and SiHa) are resistant to infection by VSV. Therefore, using *M. oleifera* in combination with VSV may increase the susceptibility of these resistant cell types to infection by VSV due to the anti-cancer properties previously demonstrated by *M. oleifera*. While not significant ($p=0.059$), pretreatment with 200 µg/ml *M. oleifera* ethanolic extract for 6 hours sensitized C4-II cells to killing by the rwt strain of VSV (Figure 2A). The *M. oleifera* ethanolic extract did not, however, increase C4-II cell killing by the rM51R-M virus following a 24 infection period. By 48 hours, differences in cell viability were not observed due to the ability of both viruses to effectively kill approximately 95% of all cells (Figure 2B).

In HeLa cells, an increasing trend in killing by rwt-VSV following a 6 hour pretreatment and a 24 hour incubation with virus was seen, but was not statistically significant (p=0.10) (Figure 3A). Furthermore, *M. oleifera* ethanolic extract did not promote the killing of cells by rM51R-M virus when infected for 24 hours (Figure 3A) or 48 hours (data not shown). Interestingly, pretreatment of SiHa cells with the methanolic extracts for 6 hours significantly enhanced the ability of rM51R-M virus to kill SiHa cells (p=0.031, Figure 3B). The aqueous, butanolic, and hydroethanolic extracts of *M. oleifera* did not influence the viability of cervical cancer cells when cells were treated alone or in combination with the VSV strains (data not shown). These results indicate that the ethanolic and methanolic extracts contain active compounds that may influence the ability of cervical cancer cells to proliferate, undergo apoptosis or other cell death mechanisms, and synergize as therapeutic agents with VSV. Therefore, subsequent studies will focus on these extracts.

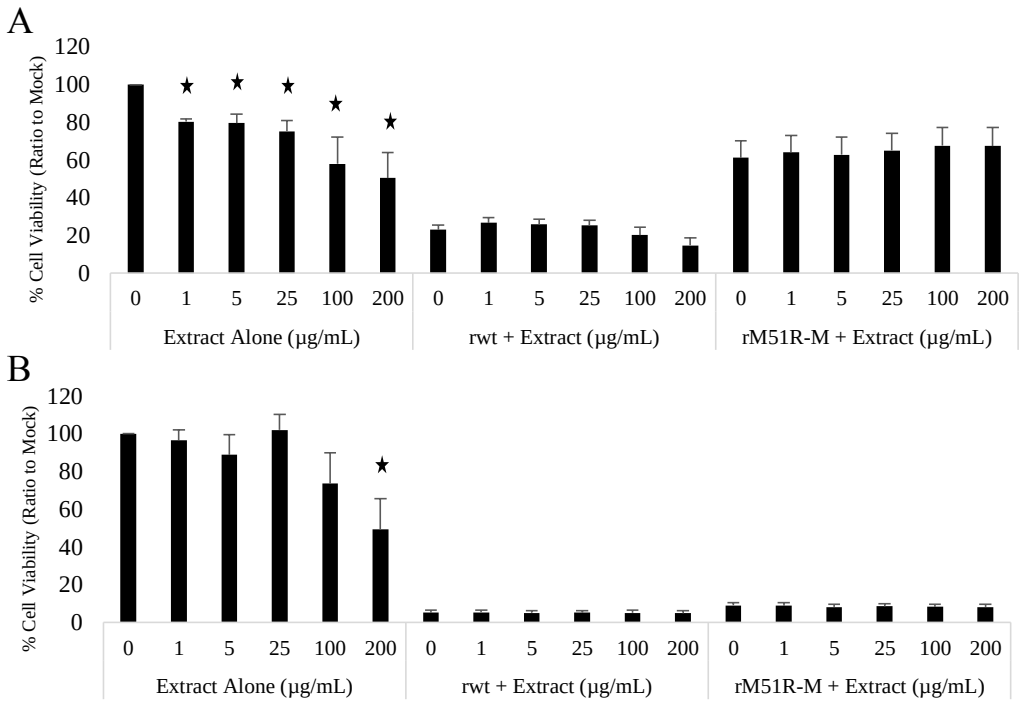

FIG. 2. *M. oleifera* ethanolic extract promotes C4-II cervical cancer cell killing alone and in combination with VSV. For extract treatment alone, C4-II cell lines were treated with extracts alone for 24 (A) or 48 (B) hours at concentrations ranging from 0-200 µg/ml. For combination treatment, C4-II cells were pretreated for 6 hours at concentrations ranging from 0 – 200 µg/ml and infected with rwt-VSV or rM51R-M-VSV at a MOI of 10 pfu/cell for 24 or 48 hours. MTT cell proliferation assays were performed to determine cell viability of wells, determined as an OD. Data shows percent cell viability as a ratio of mock-infected wells. Data are based on three experiments and show the mean percent of cell viability as a ratio to mock infected cells ± standard error. Stars indicate *p* value of <0.05 and are based on comparisons between mock-infected cells to treatments alone, rwt-VSV cells infected alone compared to rwt plus treatment, or rM51R-M-VSV cells infected alone compared to rM51R-M infected cells plus treatment.

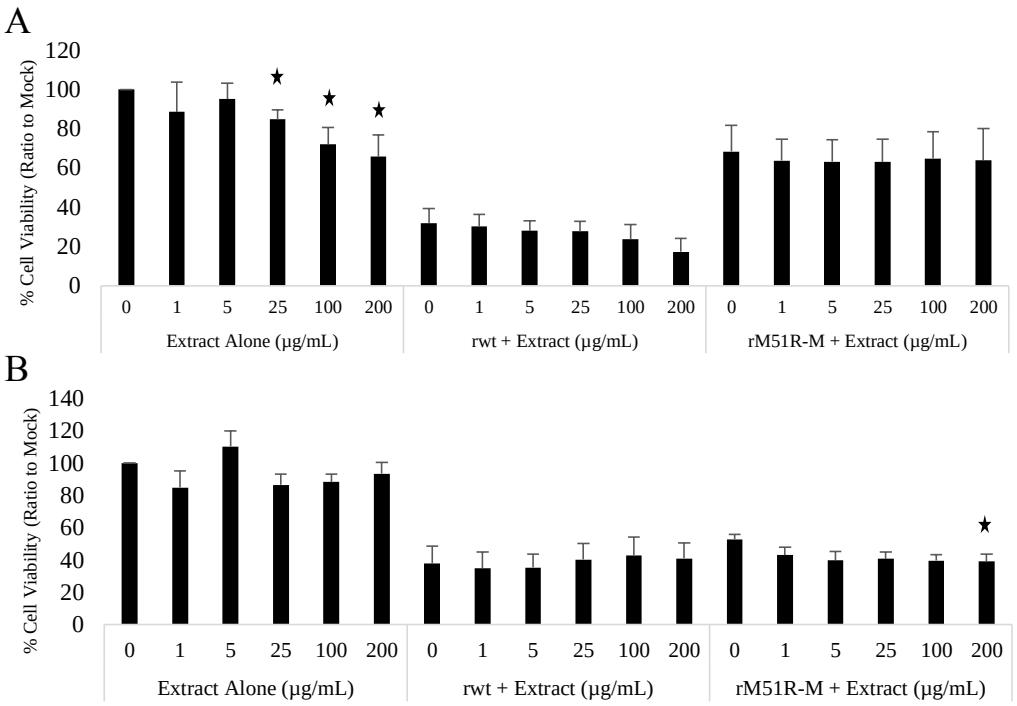

FIG. 3. *M. oleifera* ethanolic and methanolic extracts promote cervical cancer cell killing alone and/or in combination with VSV. For extract treatment alone, HeLa (A) and SiHa (B) cell lines were treated with extracts alone for 24 hours at concentrations ranging from 0-200 µg/ml. For combination treatment, cells were pretreated for 6 hours at concentrations ranging from 0 – 200 µg/ml and infected with rwt-VSV or rM51R-M-VSV at a MOI of 10 pfu/cell for 24 hours. MTT cell proliferation assays were performed to determine cell viability of wells, determined as an OD. Data shows percent cell viability as a ratio of mock-infected wells. Data are based on three experiments and show the mean percent of cell viability as a ratio to mock infected cells ± standard error. Stars indicate *p* value of <0.05 and are based on comparisons between mock-infected cells to treatments alone, rwt-VSV cells infected alone compared to rwt plus treatment, or rM51R-M-VSV cells infected alone compared to rM51R-M infected cells plus treatment.

M. oleifera increases virus replication in infected cervical cancer cells

To determine if increased cell killing by VSV strains in cervical cancer cell lines was related to virus replication in infected cells, cells were pretreated for 6 hours with varying concentrations of *M. oleifera* extracts (0, 5, 25, 100, and 200 µg/ml) and infected with rwt-GFP or rM51R-M-GFP VSV strains for 6 hours. Cells were harvested and analyzed for the

percentage of cells supporting VSV replication, and the degree of replication within each cell. Because the ethanolic and methanolic extracts promoted killing of cervical cancer cells alone or in combination with VSV, we focused on the effects of these extracts on replication on VSV in C4-II, HeLa, and SiHa cells. For these studies, cells were infected with VSV strains expressing GFP during the replication cycle. Figures 4A and 4E show that there was no difference in the percentage of C4-II and SiHa cells expressing GFP when cells were infected with VSV in the presence of increasing concentrations of the ethanolic extract. In contrast, there was a significant increase in the percentage of HeLa cells expressing GFP when treated with the ethanolic extract (p=0.049 at 25 μg/ml, Figure 4C). Furthermore, there was a significant increase in the degree of rM51R-M replication in infected C4-II and HeLa cells when treated with concentrations of ethanolic extract equivalent or greater than 25 μg/ml for C4-II cells (p=0.007), and 100 μg/ml for HeLa cells (p=0.021) (Figure 4B and 4D, respectively). Interestingly, rM51R-M virus replicated more efficiently in infected C4-II and HeLa cells as compared to the rwt virus, as shown in Figure 4B and 4D, respectively. As shown in Figure 4F, the ethanolic extract did not influence replication of VSV in SiHa cells. These results suggest that compounds present in the ethanolic extract may allow for increased replication of the mutant strain in C4-II and HeLa cells, potentially due to the anti-inflammatory effects associated with *M. oleifera* that allow for increased virus replication to occur. Furthermore, for oncolytic therapies, it is also encouraging to observe that the ethanolic extract did not negatively impact VSV replication.

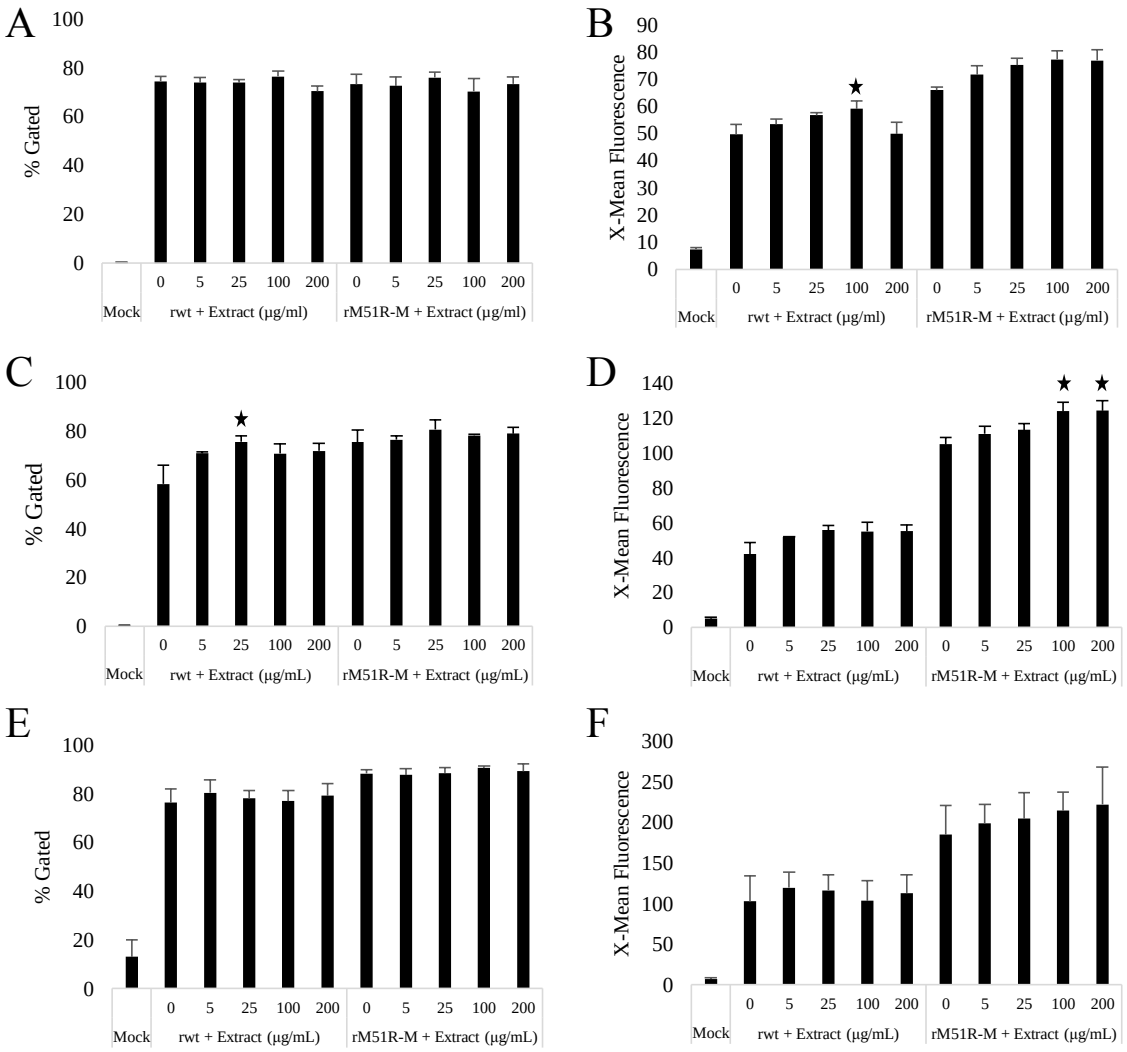

FIG. 4. *M. oleifera* ethanolic extract promotes rM51R-M-VSV replication in C4-II and HeLa cells, but does not influence virus infection of cells. C4-II, HeLa, and SiHa cells were pretreated with *M. oleifera* ethanolic extract at concentrations ranging from 0 – 200 µg/ml for 6 hours and infected with rwt-GFP or rM51R-M-GFP VSV strains for 6 hours at an MOI of 10 pfu/cell. Cells were harvested and GFP expression was determined via flow cytometry. C4-II (A), HeLa (C), and SiHa (E) pretreated with methanolic extract and infected with rwt-GFP or rM51R-M-GFP virus were examined for % Gated, or the percent of cells allowing replication in infected cells. C4-II (B), HeLa (D), and SiHa (F) cells were also examined for x-mean fluorescence, or the degree of viral replication within each infected cell. The x-mean fluorescence serves as a measurement for the degree of replication within each infected cell. Data shows the mean of three experiments ± standard error. Stars indicate significance compared to the respective mock – rwt-GFP without treatment or rM651R-M-GFP without treatment.

The methanolic extract was also examined for its potential influence on the ability of VSV to infect and replicate in C4-II, HeLa, and SiHa cells. The methanolic extract did not affect the replication of rwt virus in HeLa (Figure 5C) and SiHa (Figure 5E) cells. However, an increase in GFP expression was observed in C4-II cells infected with rwt virus (p=0.043 at the 25 μg/ml treatment, Figure 5A). In addition, the expression of GFP in rM51R-M virus in SiHa cells increased in conjunction with concentrations of 5, 100, and 200 μg/ml methanolic extract (Figure 5E). These results suggest that the methanolic extract promotes permissiveness of C4-II and SiHa cervical cancer cell to infection by rwt and rM51R-M virus, respectively.

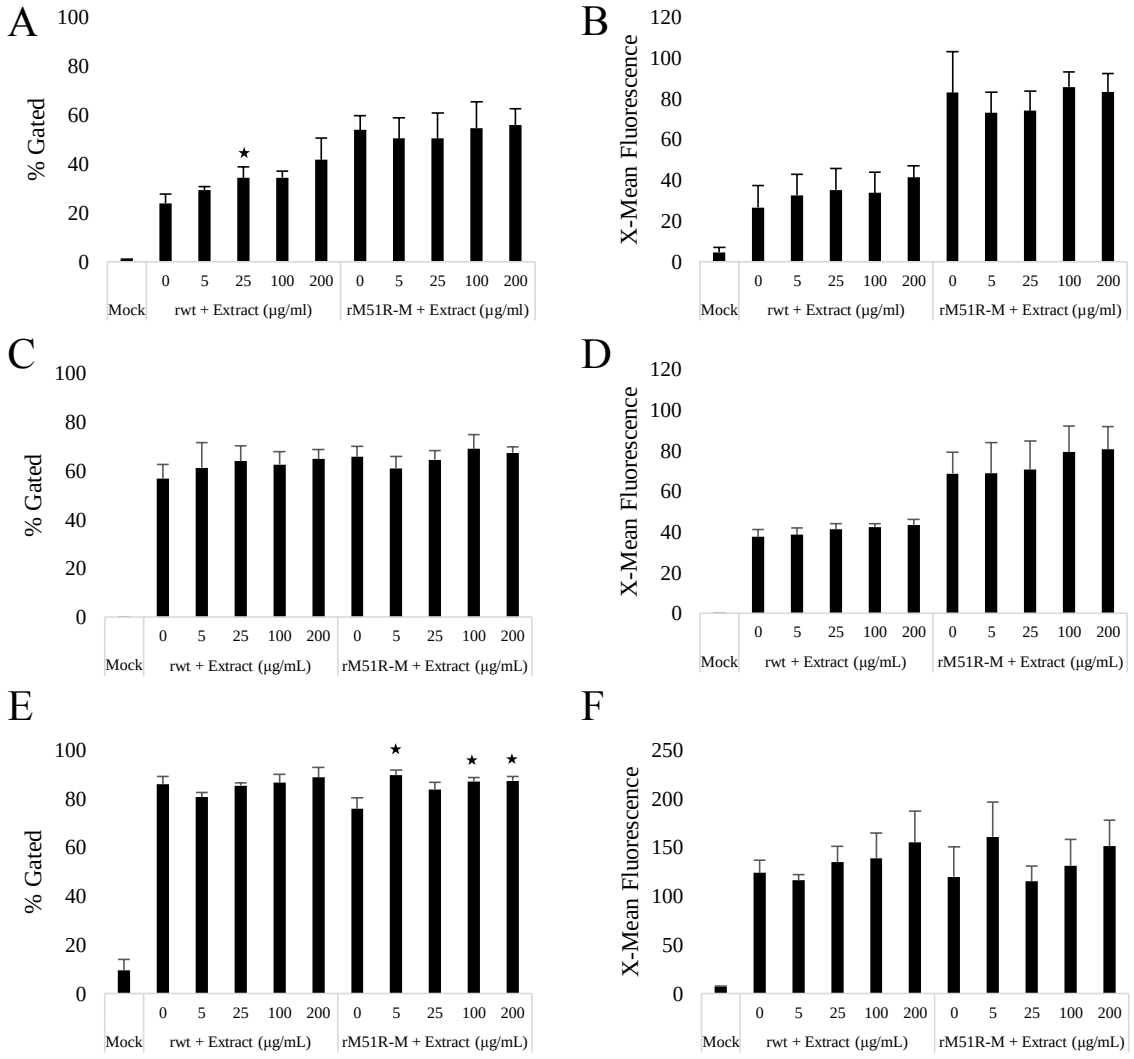

FIG. 5. The methanolic extracts promotes permissiveness of C4-II and SiHa cells to infection by VSV. C4-II, HeLa, and SiHa cells were pretreated for 6 hours with methanolic extracts at concentrations ranging from 0 – 200 µg/ml and infected with rwt-GFP or rM51R-M-GFP virus at an MOI of 10 pfu/cell for 6 hours. Cell were harvested and analyzed for GFP expression via flow cytometry. C4-II (A), HeLa (C), and SiHa (E) pretreated with methanolic extract and infected with rwt-GFP or rM51R-M-GFP virus were examined for % Gated, or the percent of cells allowing replication in infected cells. C4-II (B), HeLa (D), and SiHa (F) cells were also examined for x-mean fluorescence, or the degree of viral replication within each infected cell. Data shows mean of three experiments ± standard error. Stars signify significance of treated cells compared to cells infected without treatment.

M. oleifera ethanolic extract alters NF-κB protein levels and expression in cervical cancer cells

As shown earlier, the ethanolic extract was shown to promote killing of cervical cancer cells alone and in combination with rwt-VSV. One hypothesis is that the ethanolic extract may alter cell signaling pathways associated with cell survival and proliferation, such as the NF-κB pathway, which has previously been demonstrated to be constitutively activated in some cancer cells (3, 39). Modification of the NF-κB pathway may sensitize cells to infection and killing by VSV. To determine if the ethanolic extract alters NF-κB activation, C4-II and HeLa cells were pretreated for 6 hours with the ethanolic extract (5, 25, and 100 μg/ml) and infected with rwt or rM51R-M virus strains for 24 hours. Whole cell lysates were collected and protein expression was assessed via Western blotting. As shown in Figure 6A, total NF-κB protein levels in C4-II significantly increased when treated with 5 μg/ml (p=0.026) and 100 μg/ml (p=0.011) of the ethanolic extract. In addition, phosphorylation of NF-κB was significantly inhibited when C4-II cells were treated with 100 μg/ml of ethanolic extract (p=0.006). Total NF-κB levels decreased in a concentration-dependent manner in HeLa cells, with a significant decline (p=0.014) in expression upon treatment with 100 μg/ml ethanolic extract (Figure 6B). These results suggest that the ethanolic extract may decrease total NF-κB protein concentration in HeLa cells and activation of NF-κB in C4-II cells, which may play a role in the anti-proliferative and anti-cancer role the extract exhibited on these cells. Additionally, the increase exhibited in total NF-κB protein levels in C4-II cells may relate to upstream activation of other cell signaling pathways that may promote to the cytotoxic effect seen by the ethanolic extract.

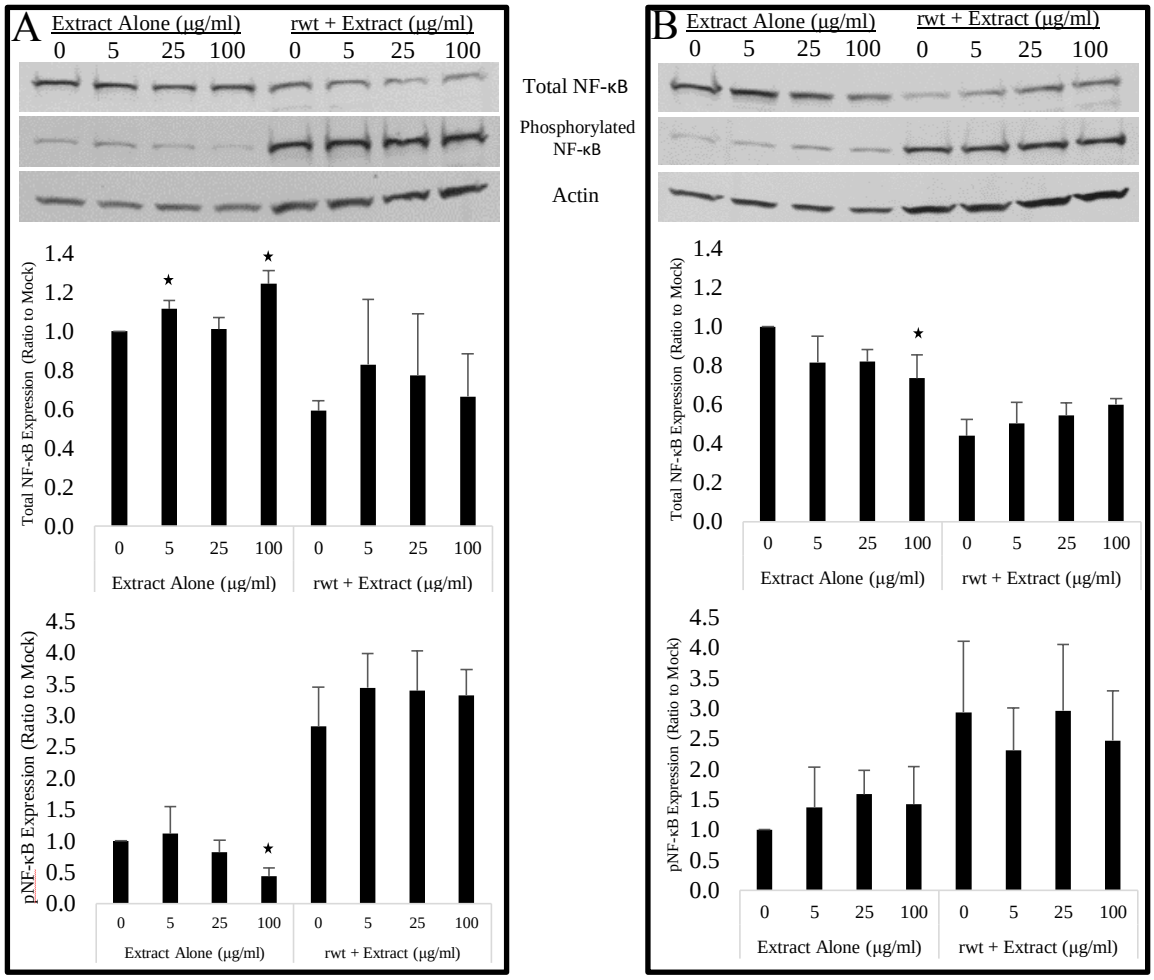

FIG. 6. *M. oleifera* ethanolic extract effects NF-κB levels and expression in C4-II and HeLa cells. C4-II (A) and HeLa (B) cells were pretreated for 6 hours with ethanolic extract and subsequently infected with rwt-VSV at an MOI of 10 pfu/cell for 24 hours. Cell lysates were collected and total and phosphorylated NF-κB protein expression was determined via Western blotting. Expression was determined via densitometry, and expression is shown as a ratio of mock cell. Data represents the mean of three experiments ± standard error. Stars indicate statistical significance ($p<0.05$).

M. oleifera ethanolic extract alters Akt protein levels and expression in cervical cancer cells

The Akt pathway has also been implicated in the survival and proliferation of cancer cells (43). Therefore, the decrease in proliferation and increase in killing of C4-II and HeLa cells by *M. oleifera* may be due to alterations in the Akt pathway. C4-II and HeLa cells were pretreated for 6 hours with the ethanolic extract (5, 25, and 100 μg/ml) and infected for 24 hours with rwt-VSV to determine if the Akt pathway was implicated in the anti-proliferative and anticancer effects seen with and without rwt virus. Whole lysates were collected and total and phosphorylated Akt levels were determined via Western blotting. In C4-II cells, a significant decrease (p=0.041) in total Akt levels at the 5 μg/ml treatment and a significant increase in phosphorylated Akt levels at concentrations of 25 and 100 μg/ml ethanolic extract were observed (Figure 7A). While there was no influence of the ethanolic extract on Akt expression in HeLa, an overall increasing trend in total Akt levels in rwt-infected HeLa cells was seen (Figure 7B). Additionally, Akt expression was not altered in rwt-infected C4-II cells. Results suggest that at higher concentrations, the ethanolic extract promotes activation of Akt in C4-II cells following treatment alone which may promote downstream anti-proliferative or apoptotic signaling pathways not examined. Additionally, pre-incubation with the ethanolic extract increases total expression, but not activation of, Akt levels in rwt-infected HeLa cells. Further analysis of the effect of the ethanolic extract on Akt expression in rwt-infected cells is needed to determine statistical significance.

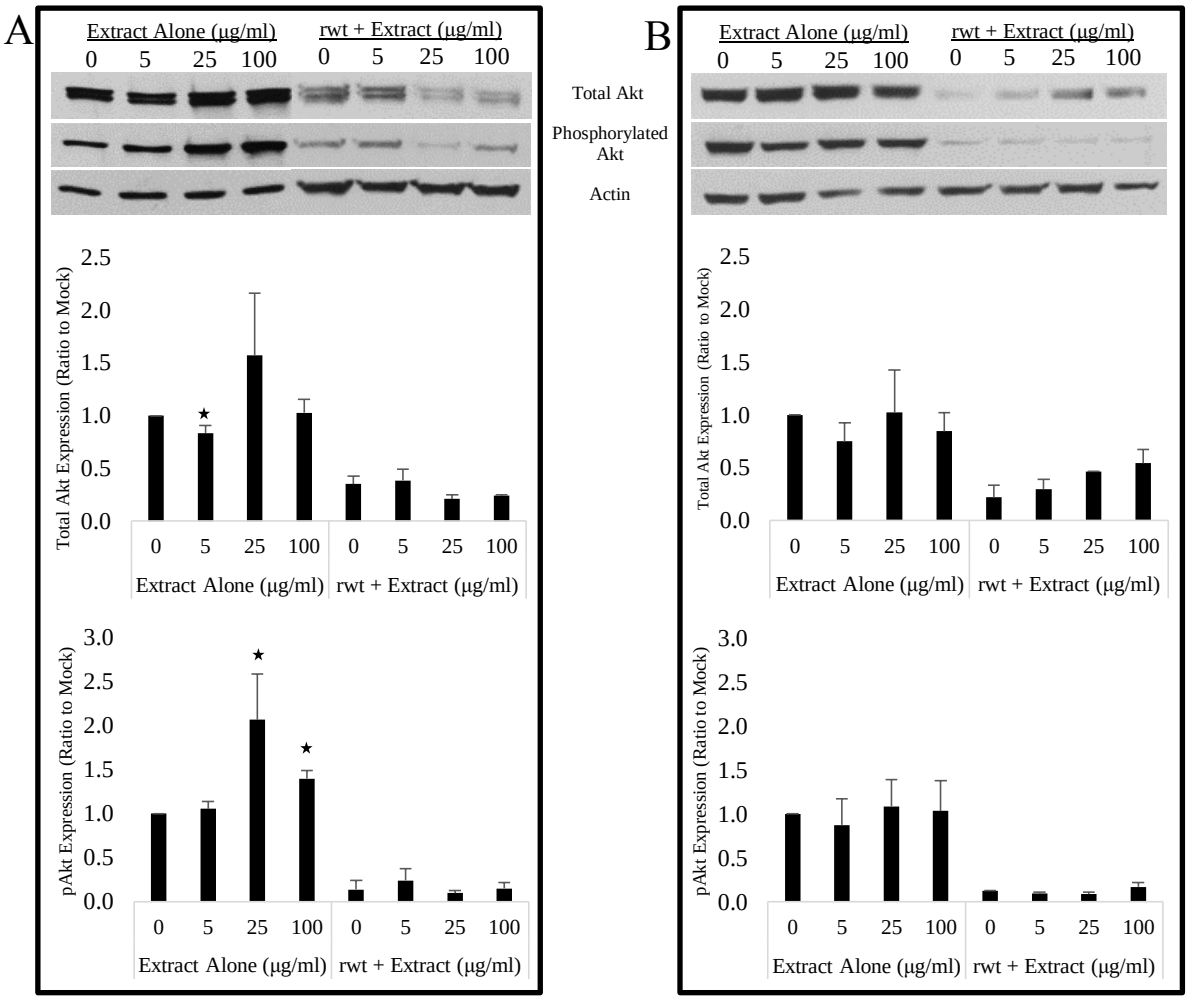

FIG. 7. *M. oleifera* ethanolic extract alters Akt levels in cervical cancer cells. C4-II (A) and HeLa (B) cells were pretreated for 6 hours and infected with rwt-VSV at an MOI of 10 pfu/cell for 24 hours. Whole cell lysates were collected and total and phosphorylated Akt levels were determined via Western blotting. Expression was determined via densitometry, and expression is shown as a ratio of mock cell. Data represents the mean of three experiments ± standard error for treatment alone samples. The mean of two experiments ± standard error are shown for samples infected with rwt virus. Stars indicate statistical significance ($p<0.05$).

M. oleifera ethanolic extract alters expression of Bcl-2 family members in C4-II and HeLa cells

The Bcl-2 family of proteins, which has been implicated in playing a role in different types of cancer, can be activated downstream of different signaling pathways such as NF-κB and Akt. Bax and Bcl-XL, members of the Bcl-2 family, have previously been identified as a pro-apoptotic and anti-apoptotic factors, respectively (43). To determine if Bax or Bcl-XL expression contributed to the cell killing of C4-II and HeLa cells treated with the ethanolic extract alone or in combination with the rwt virus, cells were pretreated for 6 hours with the ethanolic extract (5, 25, and 100 μg/ml) and infected with rwt virus for 24 hours. Whole cell lysates were collected and Bax and Bcl-XL expression was determined via Western blotting. Bax expression significantly decreased (p=0.012 at 100 μg/ml) in a concentration-dependent manner in C4-II cells following treatment alone, but significantly increased (p=0.033) in rwt-infected C4-II cells pretreated with 100 μg/ml of ethanolic extract (Figure 8A). However, Bax expression in HeLa cells was not altered under treatment alone or treatment plus rwt-infection conditions (Figure 8B). The ethanolic extract significantly inhibited Bcl-XL expression in C4-II cells, and significantly promote Bcl-XL expression in HeLa cells (Figure 9A and 9B). These results show that the ethanolic extract may promote Bax-mediated cell killing of rwt-infected C4-II cells. Decreased Bcl-XL activation in treated C4-II cells may increase their susceptibility to apoptosis via other cell signaling pathways. The increase in Bcl-XL activation in HeLa cells suggests the ethanolic extract may promote cell killing via different mechanisms than those in C4-II cells.

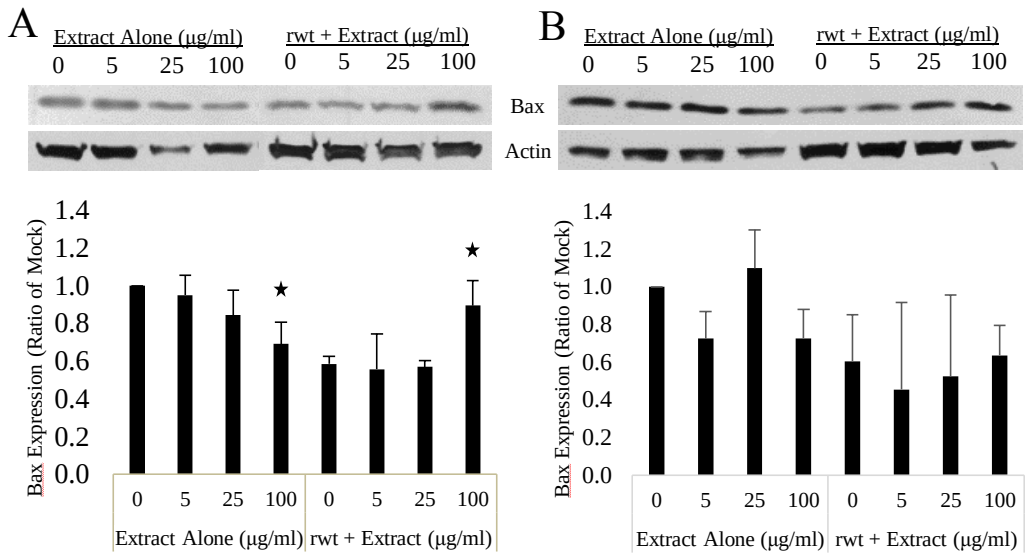

FIG. 8. *M. oleifera* ethanolic extract influences Bax expression in C4-II cells, but not in HeLa cells. C4-II (A) and HeLa (B) cells were pretreated with ethanolic extract at concentrations of 5, 25, or 100 µg/ml for 6 hours and were infected with rwt-virus for 24 hours. Whole cell lysates were collected, and Bax expression was determined via Western blotting. Expression was determined via densitometry, and expression is shown as a ratio of mock cell. Data shown is the mean of three experiments ± standard error. Stars indicate statistical significance.

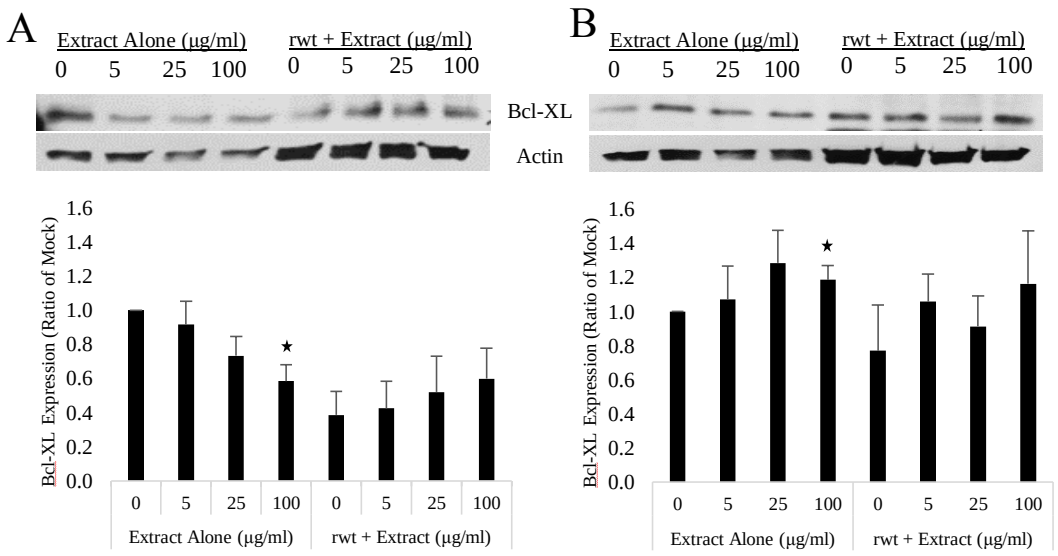

FIG. 9. *M. oleifera* ethanolic extract alters Bcl-XL expression in C4-II and HeLa cells. C4-II (A) and HeLa (B) cells were pretreated with ethanolic extract at concentrations of 5, 25, or 100 µg/ml for 6 hours and were infected with rwt-virus for 24 hours. Whole cell lysates were collected, and Bcl-XL expression was determined via Western blotting. Expression was determined via densitometry, and expression is shown as a ratio of mock cell. Data shown is the mean of three experiments ± standard error. Stars indicate statistical significance ($p<0.05$).

M. oleifera ethanolic extract alters STAT1 activation in cervical cancer cells

An essential feature of oncolytic therapy is the antiviral response, whose presence or absence may influence the outcome of therapies in virus-infected cancer cells. Previous studies have shown that the antiviral (or type I IFN) response is often defective in cancer cells due to mutations that arise in these cells to support their own survival and growth (22). Taking advantage of these and other cell signaling pathway mutations is one way in which VSV may demonstrate its increased ability to infect cancer cells. Additionally, *M. oleifera* may also promote survival and replication of VSV in infected cells by inhibiting the antiviral response. To determine if *M. oleifera* influences the type I IFN antiviral response, C4-II and

HeLa cells were pretreated with the ethanolic extract, and SiHa cells with the methanolic extract, for 6 hours. Cells were infected with rwt and rM51R-M VSV strains or treated with type I IFN for 24 hours. Whole cell lysates were collected and total and phosphorylated STAT1 expression was determined via Western blotting. STAT1 is an essential upstream transcription factor in the IFN response pathway. Its expression and activation can be measured to examine the influence of *M. oleifera* on the antiviral response. As shown in Figures 10 and 11, extract alone did not alter levels of total or phosphorylated STAT1 in C4-II or HeLa cells. However, it did significantly decrease the expression of total STAT1 induced by IFN treatment and infection with rwt virus in both cell types. In SiHa cells, interestingly, the methanolic extract enhanced levels of total and phosphorylated STAT1 (Figure 12). In addition, it promoted the increase in total and phosphorylated levels in conjunction with IFN treatment. These results indicate that in C4-II and HeLa cells, the ethanolic extract may decrease the type I IFN response pathway by reducing expression of total STAT1. In contrast, the methanolic extract enhances the antiviral response in SiHa cells.

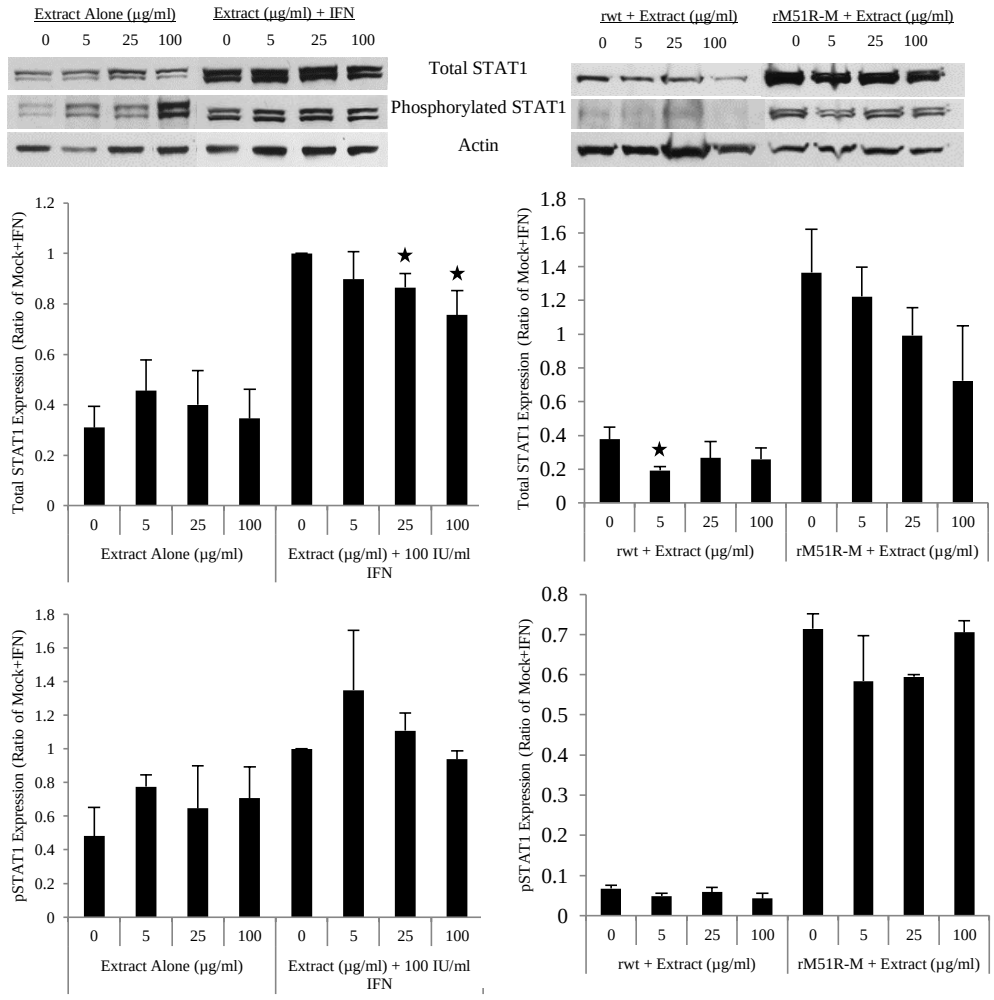

FIG. 10. *M. oleifera* ethanolic extract alters STAT1 expression in C4-II cervical cancer cells. C4-II cells were pretreated with ethanolic extract at concentrations of 5, 25, or 100 µg/ml for 6 hours and were infected with rwt or rM51R-M VSV at an MOI of 10 pfu/cell or treated with 100 IU/ml type I IFN for 24 hours. Whole cell lysates were collected, and total and phosphorylated STAT1 expression was determined via Western blotting. Expression was determined via densitometry, and expression is shown as a ratio of IFN-treated mock cells. Data shown for treatment alone or IFN-treated cells is the mean of three experiments ± standard error. Data for rwt or rM51R-M infected cells is the mean of two experiments ± standard error, excluding total STAT1 expression for rwt-infected cells which is based on three experiments ± standard error. Stars indicate statistical significance, and are based on comparisons between untreated cells and ethanolic extract-treated conditions.

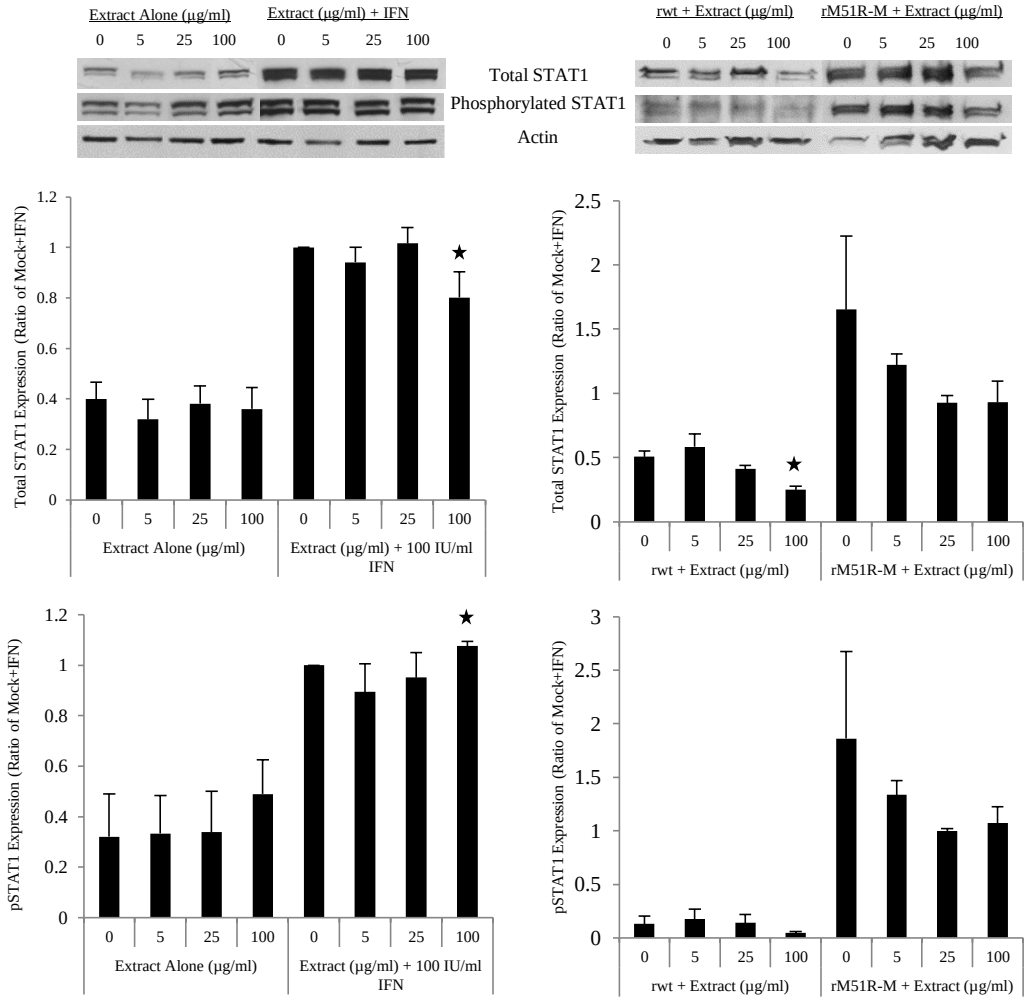

FIG. 11. *M. oleifera* ethanolic extract alters STAT1 expression in HeLa cervical cancer cells. HeLa cells were pretreated with ethanolic extract at concentrations of 5, 25, or 100 µg/ml for 6 hours and were infected with rwt or rM51R-M VSV at an MOI of 10 pfu/cell or treated with 100 IU/ml type I IFN for 24 hours. Whole cell lysates were collected, and total and phosphorylated STAT1 expression was determined via Western blotting. Expression was determined via densitometry, and expression is shown as a ratio of IFN-treated mock cells. Data shown for treatment alone or IFN-treated cells is the mean of three experiments ± standard error. Data for rwt or rM51R-M infected cells is the mean of two experiments ± standard error, excluding total STAT1 expression for rwt-infected cells which is based on three experiments ± standard error. Stars indicate statistical significance, and are based on comparisons between untreated cells and ethanolic extract-treated conditions.

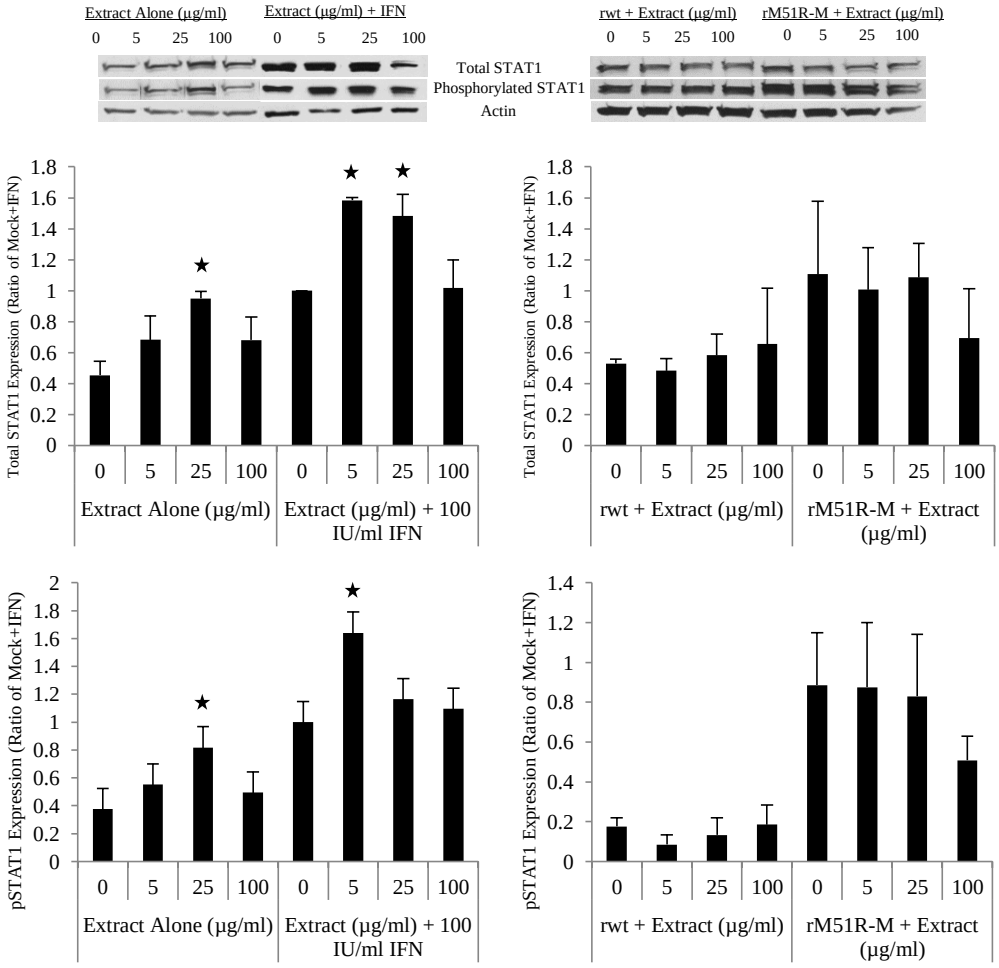

FIG. 12. *M. oleifera* methanolic extract alters STAT1 expression in SiHa cervical cancer cells. SiHa cells were pretreated with methanolic extract at concentrations of 5, 25, or 100 µg/ml for 6 hours and were infected with rwt or rM51R-M VSV at an MOI of 10 pfu/cell or treated with 100 IU/ml type I IFN for 24 hours. Whole cell lysates were collected, and total and phosphorylated STAT1 expression was determined via Western blotting. Expression was determined via densitometry, and expression is shown as a ratio of IFN-treated mock cells. Data shown for treatment alone or IFN-treated cells is the mean of three experiments ± standard error. Data for rwt or rM51R-M infected cells is the mean of two experiments ± standard error. Stars indicate statistical significance, and are based on comparisons between untreated cells and ethanolic extract-treated conditions.

M. oleifera ethanolic extract stimulates immune cell activation

Our results indicate that *M. oleifera* exerts a direct effect on killing of cervical cancer cells alone and in combination with VSV. However, another crucial aspect of oncolytic therapies is the ability of therapies to promote anti-tumor immunity. Previous studies have shown that *M. oleifera* has anti-inflammatory and anti-cancer properties (16). Therefore, the ability of *M. oleifera* to modulate the immune response may promote clearance of VSV-infected cervical cancer cells and induce anti-tumor immunity. To determine the effect of *M. oleifera* on stimulation of immune cells, PBMCs were isolated from whole blood and pretreated for two hours with varying concentrations of the ethanolic extract (5, 25, and 100 μg/ml). PBMCs were infected with rwt or rM51R-M VSV or treated with 500 ng/ml LPS for 20 hours. Supernatants were collected to determine levels of pro-inflammatory cytokine production, and PBMCs were harvested and labeled with antibodies to identify activation markers on different immune cell components. The costimulatory molecule, CD83 on different subsets of DC was labelled (Figure 13A and B) and levels of expression greater than background are indicative of DC subset activation. First of all, LPS, rwt and rM51R-M viruses stimulated pDC as indicated by increased levels of CD83 on the surface of these cells (Figure 13A). In addition, the ethanolic extract significantly increased pDC maturation in conjunction with LPS and rM51R-M virus (100 μg/ml treatment, $p<0.05$). mDC were not as responsive to LPS and virus as pDC, but 100 μg/ml of ethanolic extract significantly increased the stimulation of mDC in conjunction with rM51R-M virus (Figure 13B). Furthermore, although LPS, rwt and rM51R-M virus stimulated both NK and T cells, the ethanolic extract did not alter the activation of these immune cells as indicated by lack of changes in the expression of the NK and T cell activation marker, CD69, as compared to

background levels (Figure 13C & D). These data indicate that *M. oleifera* ethanolic extract stimulates activation of DC subsets following infection by rM51R-M. Because this mutant virus does not inhibit the antiviral response, the use of *M. oleifera* in combination with VSV may promote induction of an antiviral response through increased production of IFN by pDC and therefore promote the clearance of virus-infected cancer cells through increased activation of mDC which have the potential to promote activation of other key immune cells.

In addition to the stimulation of immune cells, various cytokines are secreted by PBMCs under different conditions to promote a pro-inflammatory environment. IL-6 and TNF are produced by immune cells including DC in response to various stimuli including viral infections, and therefore can be measured to examine the immune response following virus infection. To determine if *M. oleifera* ethanolic extract influenced cytokine production, ELISAs were used to determine IL-6 and TNF levels of the supernatants collected from PBMCs as previously described. LPS significantly increased IL-6 levels in PBMCs compared to mock and VSV-infected PBMCs (Figure 14A). In conjunction with LPS, the ethanolic extract significantly increased IL-6 and TNF levels at the 100 μg/ml treatment (Figure 14C & D). Furthermore, rM51R-M infected PBMCs exhibited significantly higher IL-6 levels following treatment with 100 μg/ml ethanolic extract (p=0.044, Figure 14C). In contrast, the ethanolic extract inhibited IL-6 production in rwt-infected PBMCs (Figure 14C). LPS and rM51R-M data correlates with increased DC stimulation as previously demonstrated, and support the role of *M. oleifera* in its ability to induce antiviral immunity against VSV-infected cancer cells.

M. oleifera methanolic extract alters IL-6 and TNF expression in LPS, but not VSV-infected cells

Our results indicate that there are distinct differences between the ethanolic and methanolic extracts of *M. oleifera*. To determine if these extracts differed in their ability to stimulate immune cells, PBMCs were isolated from whole blood and pretreated with varying concentrations of the methanolic extract (5, 25, and 100 μg/ml). PBMCs were infected with rwt or rM51R-M VSV or treated with 500 ng/ml LPS for 20 hours. Supernatants were collected, and IL-6 and TNF cytokine production was determined using ELISAs. The methanolic extract increased IL-6 and TNF production at the 100 μg/ml treatment in LPS-treated cells (Figure 14E & F). However, the methanolic extract did not influence cytokine production in VSV-infected cells. These results suggest that the methanolic extract promotes activation of an immune response against LPS, but does not influence cytokine production in virus-infected cells.

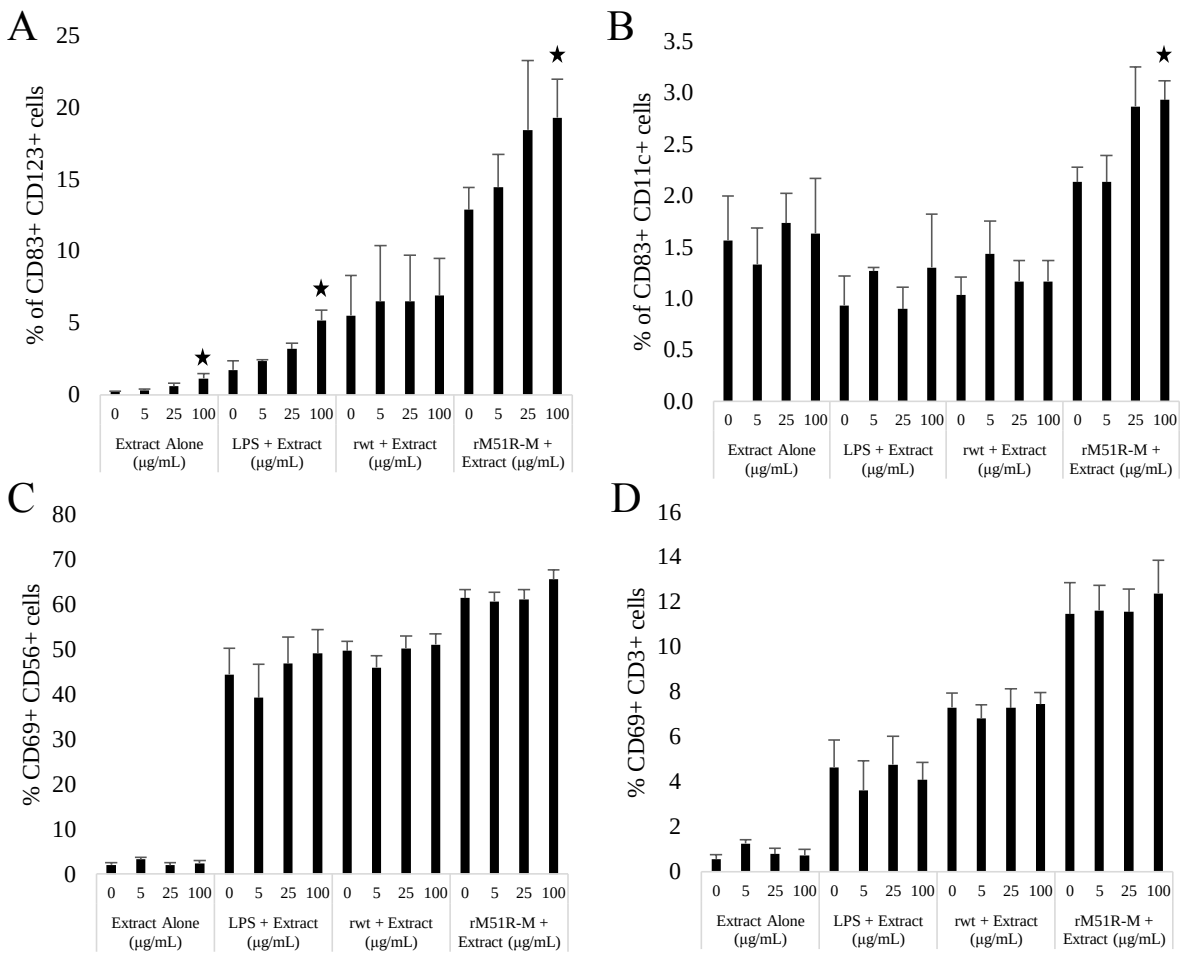

FIG. 13. *M. oleifera* ethanolic extract stimulates maturation of DC cell subsets, but not NK or T-cells. PBMCs isolated from whole blood were pretreated for 2 hours with 5, 25, or 100 µg/ml ethanolic extract and infected with rwt or rM51R-M VSV strains at an MOI of 3 pfu/cell or treated with 500 ng/ml of LPS. The stimulation of pDC (A), mDC (B), NK (C), and T (D) cells was determined via flow cytometry with the use of fluorescently-labeled antibodies against CD markers. Data shown are the mean of three experiments ± standard error. Stars indicate statistical significance using one-tailed t-tests, and are based on comparisons between untreated, LPS-treated, rwt, or rM51R-M infected cells to their ethanolic extract-treated cells, respectively.

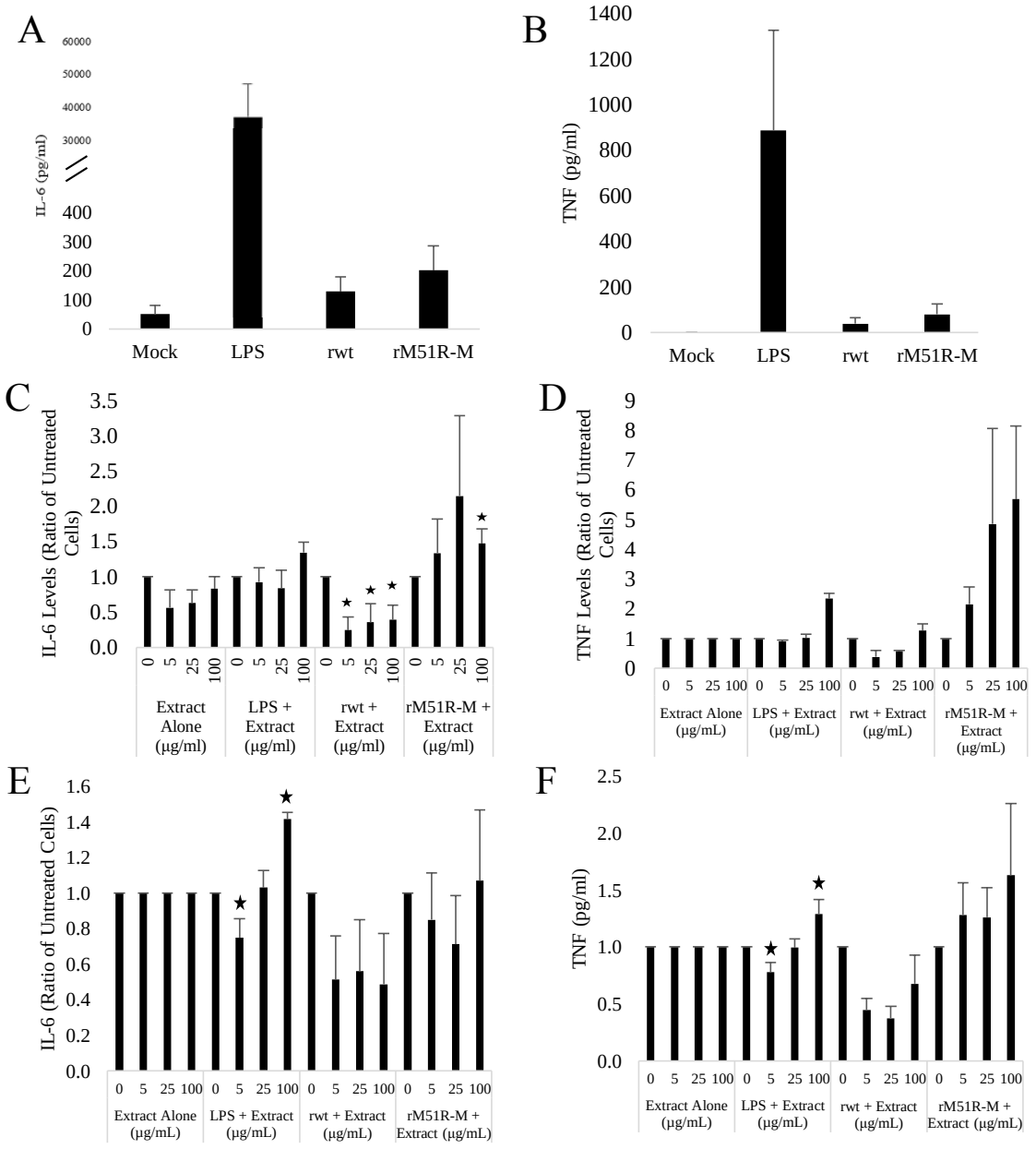

FIG. 14. *M. oleifera* ethanolic and methanolic extracts alter cytokine expression in PBMCs. PBMCs were isolated from whole blood and pretreated for 2 hours with 5, 25, or 100 µg/ml ethanolic or methanolic extract. Cells were infected with rwt or rM51R-M VSV strains at an MOI of 3 pfu/cell or treated with 500 ng/ml LPS for 20 hours. Supernatants were collected and IL-6 and TNF levels were determined with ELISAs for the ethanolic (C & D) and the methanolic (E & F) extracts. Data shown in A and B are cytokine levels of untreated cells in pg/ml. Data shown are cytokine levels as a ratio of respective untreated cells and are the mean of three experiments ± standard error.

M. oleifera ethanolic extract alters cytokine production in PBMC-HeLa cell co-culture

So far, experiments have shown that *M. oleifera* has a direct impact on cervical cancer cell killing both alone and in combination with VSV and promotes activation of an immune response as indicated by experiments with PBMCs. However, the direct relationship between cervical cancer cells and PBMCs has not been examined. To better identify the role of *M. oleifera* in promoting clearance of virus-infected cancer cells by immune cells, PBMCs were isolated from whole blood and incubated directly with HeLa cells in culture. As a control, HeLa and PBMCs were incubated individually for comparison with co-culture conditions. Cells were pretreated with the ethanolic extract (25 and 100 μg/ml) for 2 hours and infected with rwt or rM51R-M VSV strains for 24 hours. Supernatants were collected and IL-6, TNF, and IFNγ levels were determined using ELISAs. As shown in Figure 15, rwt virus inhibited IL-6 production in untreated HeLa and PBMCs, whereas rM51R-M virus promoted IL-6 production in untreated PBMCs and HeLa cells. Interestingly, addition of the ethanolic extract further suppressed production of IL-6 in rwt-infected PBMCs and rM51R-M-infected HeLa cells (Figure 15). IL-6 and TNF production was not altered in HeLa-PBMC co-cultures in the presence of the ethanolic extract and VSV, but a decrease in IFNγ levels at the 100 μg/ml ethanolic extract treatment in rM51R-M infected co-cultures was exhibited (Figures 15 & 16). These results suggest that the ethanolic extract inhibits production of pro-inflammatory cytokines in individual cancer and immune cell populations infected with VSV. Furthermore, the ethanolic does not influence production of pro-inflammatory cytokines in HeLa-PBMC co-cultures infected with VSV. These results demonstrate that *M. oleifera* ethanolic extract promotes an anti-inflammatory state in individual populations

which may support the ability of VSV to efficiently infect and replicate in infected cells. Furthermore, *M. oleifera* does not negatively impact the ability of immune cells to induce an immune response following infection by VSV, as demonstrated by co-culture results.

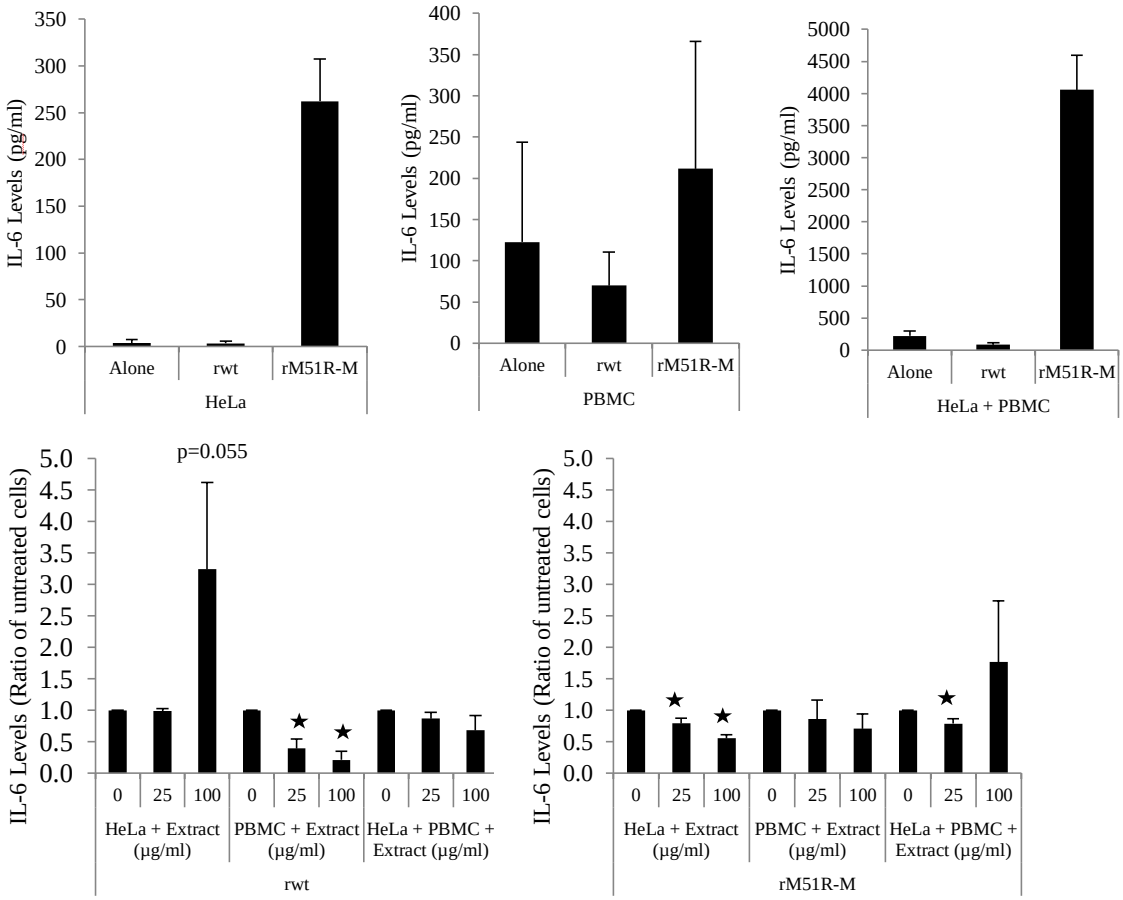

FIG. 15. *M. oleifera* ethanolic extract alters IL-6 cytokine expression in HeLa-PBMC co-culture. PBMCs were isolated from whole blood and were incubated at $2x10^6$ cells/ml with HeLa cells at $5x10^5$ cells/ml. Cells were pretreated 25 or 100 µg/ml ethanolic extract for 2 hours, and infected with rwt or rM51R-M VSV strains at an MOI of 3 pfu/cell for 24 hours. Supernatant was collected and IL-6 cytokine production was examined with ELISAs. Data shown are cytokine levels in pg/ml for untreated cells, and the ratio of treated cells to their untreated condition are shown for comparison. Data shown are the mean of three experiments ± standard error.

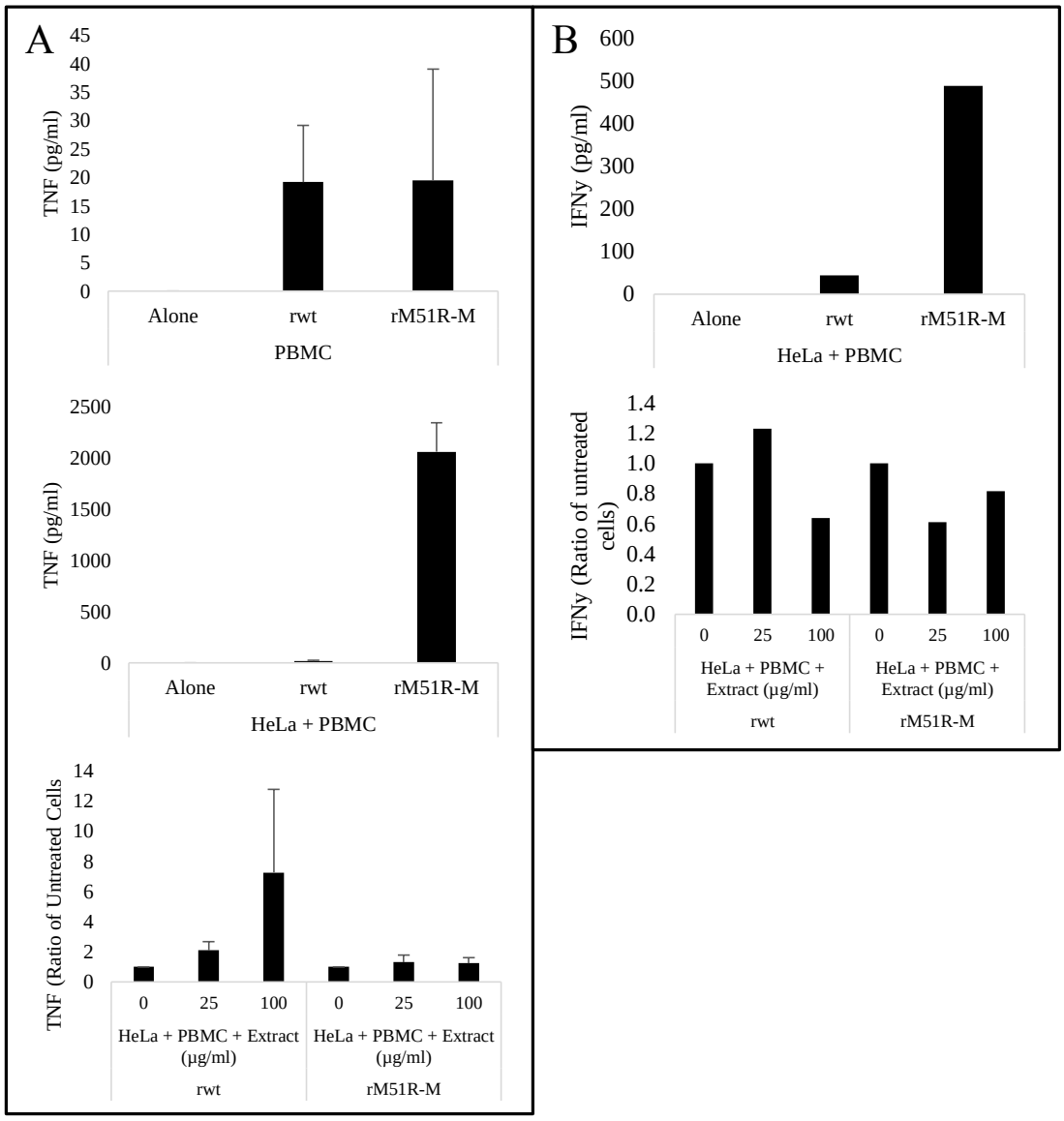

FIG. 16. *M. oleifera* ethanolic extract alters TNF and IFNγ cytokine expression in HeLa-PBMC co-culture. PBMCs were isolated from whole blood and were incubated at $2x10^6$ cells/ml with HeLa cells at $5x10^5$ cells/ml. Cells were pretreated 25 or 100 µg/ml ethanolic extract for 2 hours, and infected with rwt or rM51R-M VSV strains at an MOI of 3 pfu/cell for 24 hours. Supernatant was collected and TNF (A) and IFNγ (B) cytokine production was examined with ELISAs. Data shown are cytokine levels in pg/ml for untreated cells, and the ratio of treated cells to their untreated condition are shown for comparison. Data shown are the mean of three experiments ± standard error, except for IFNγ production, which is based on one experiment.

Discussion

Oncolytic virus therapy serves as a promising option for the treatment of various types of cancer, including cervical cancer. Numerous studies have demonstrated the ability of oncolytic viruses such as VSV to efficiently infect and replicate in cancer cells due to random mutations that promote their susceptibility to infection (37, 72). The two strains of VSV used in our research vary in their pathogenicity. The rwt virus is capable of inhibiting host gene expression, which includes inhibition of the type I IFN antiviral response. The rM51R-M mutant strain, however, is defective at inhibiting the antiviral response and thus serves as a safer alternative for use in cancer treatment. Furthermore, rM51R-M infected cells remain capable of initiating an inflammatory and antiviral response to infection by the virus (4). While VSV has demonstrated its effectiveness as an oncolytic agent in certain types of cancer cells, other cancer cells have demonstrated increased resistance to infection (4). Therefore, the use of natural compounds in combination with VSV may promote increased killing of these resistant cell types and may serve as a safer alternative to using traditional treatment options such as chemotherapy, radiation therapy, and surgery (41, 62). *M. oleifera* has previously been shown to have anti-inflammatory and anticancer properties and therefore, when used in combination with VSV, serves as a promising treatment option for cervical cancer (16, 20, 90). The results of this study found that when used in combination with VSV, *M. oleifera* increased cervical cancer cell killing by altering cell

signaling pathways that promote their susceptibility to infection by VSV. Additionally, *M. oleifera* stimulated the activation of key immune cells, suggesting it may promote clearance of virus-infected cancer cells.

Previous results in our lab examining the effect of curcumin on the susceptibility of prostate cancer cells to VSV found that co-treatment of curcumin with VSV did not augment the killing of cancer cells. However, pretreatment of PC3 prostate cancer cells for 6 hours with curcumin promoted killing of PC3 prostate cancer cells by VSV (data not shown). Therefore, our experiments focused on a 6 hour pretreatment with the extracts to allow for adequate time for potential activation of cellular pathways in response to compounds present in the extracts prior to infection. Of the five extracts of *M. oleifera* examined – aqueous, butanolic, ethanolic, hydroethanolic, and methanolic – the ethanolic and methanolic extracts were shown to promote cell killing when used in combination with VSV. Specifically, the ethanolic extract sensitized C4-II and HeLa cells to killing by rwt virus while the methanolic extract promoted SiHa cell killing by the rM51R-M virus. Additionally, the ethanolic extract was shown to inhibit C4-II and HeLa cell proliferation. These differing responses to the extracts used suggest that the ethanolic and methanolic extracts differ in their chemical compositions. In addition to the differences in compounds present in the extracts, genetic variations between the cervical cancer cells examined may also play a role their response to treatment with the extracts. For example, C4-II and HeLa cells are transformed with HPV-18 DNA, whereas SiHa cells are transformed with HPV-16 DNA. It is also essential to note these cells vary in additional mutations they have acquired to develop the cancerous phenotype (11, 65, 73, 76, 96). The variations in responses between the cervical cancer cells examined are important to consider in the context of clinical trials and how responses to the

combined treatment with VSV and *M. oleifera* may vary between individuals. Therefore, it is important to understand the underlying mechanisms and immune responses involved in this combined therapy to develop a stronger understanding of potential responses to treatment.

The ethanolic extract was shown to inhibit C4-II and HeLa cell proliferation. Furthermore, the ethanolic extract was also shown to promote C4-II and HeLa cell killing when used alone and in combination with rwt virus. The effect of the ethanolic extract on cell killing is more apparent following administration of the ethanolic extract at higher concentrations. Examination of the cell signaling pathways, to potentially identify mechanisms within treated and infected cells that promote these anticancer and anti-proliferative properties, highlighted some interesting characteristics that differed between C4-II and HeLa cells. First, it is important to note the significant increase in total NF-κB levels, and the significant decrease in phosphorylated NF-κB at the 100 μg/ml treatment, in C4-II cells following treatment alone. These results indicate that the ethanolic extract decreases activation of NF-κB in these cells. In contrast, HeLa cells treated with the ethanolic extract exhibited a significant decrease in total NF-κB levels, with no discernable effect on phosphorylated NF-κB levels. Therefore, in HeLa cells, the ethanolic extract may modulate the NF-κB pathway by decreasing total NF-κB levels. Berkovich et al. (16) showed that *M. oleifera* inhibited activation of the NF-κB pathway in pancreatic cancer cells, but at much higher concentrations than those used in our studies. These results suggest that the decreases in NF-κB total and/or phosphorylated levels contribute to inhibiting cell survival and proliferation in C4-II and HeLa cells. Furthermore, these data also support the role of *M. oleifera* as an anti-inflammatory agent, due to the suppression of the NF-κB pathway.

Compared to cells treated with the ethanolic extract alone, phosphorylated NF-κB levels were higher in rwt-infected cells pretreated with the ethanolic extract. Additionally, a non-significant increase in total NF-κB levels was exhibited in rwt-infected HeLa cells. Previous studies have shown that VSV promotes the activation of NF-κB to support its own survival and replication in cells (79). Decreased NF-κB activation has been shown to attenuate the cytotoxic effects of VSV during infection. In addition, the maintenance of NF-κB levels may be associated with the role of this pathway in the survival of the rwt virus in infected cells. While decreased NF-κB activation may promote cell killing by *M. oleifera*, increases in this pathway may be beneficial for VSV infection.

M. oleifera ethanolic extract increased Akt activation in C4-II cells at the 100 μg/ml treatment, but did not influence Akt levels in HeLa cells. Previous studies have shown that both NF-κB and Akt pathways are often upregulated or constitutively activated in cancer cells to support their continuous growth and proliferation (43). However, the significant increase in Akt activation in C4-II cells following treatment alone suggests pathways other than the Akt pathway may be responsible for the inhibition of cell proliferation and enhancement of cell killing we observed. While Akt is most often associated with cell survival and proliferation, studies have demonstrated that increased activation of Akt has been associated with increased cell death due to oxidative stress signals (61). Additionally, Nogueira et al. (70) demonstrated that increased activation of Akt may be correlated with cell cycle arrest. Therefore, the inhibition of cell proliferation demonstrated in cells pretreated with the ethanolic extract may relate to hyperactivation of Akt exhibited in C4-II cells. This does not explain the reason as to why the ethanolic extract inhibited HeLa cell proliferation, where Akt levels were not altered following treatment. Seeing this variation in results

between these two cell lines further supports the implications that these two cell lines differ in their own mutations and their responses to treatment with *M. oleifera*.

While only based on two experiments, the *M. oleifera* ethanolic extract increased total Akt levels in rwt-infected HeLa cells, which correlates with the increase in total NF-κB levels. This supports the observation that increased NF-κB activation may be necessary in HeLa cells to promote increased cell killing by the rwt virus. The opposite effect is seen in C4-II cells, in which total Akt levels decreased in rwt-infected cells. The decrease in Akt levels in C4-II further supports the role of *M. oleifera* as an anti-cancer and anti-proliferative agent. Previous studies have shown that decreases in Akt levels promotes an anti-tumor environment (2). Therefore, it is possible that the inhibition of Akt expression in rwt-infected and *M. oleifera* treated C4-II cells contributes to the promotion of the cell death we observed.

The effect of the *M. oleifera* ethanolic extract on apoptotic factors was also examined. Apoptotic factors, such as those of the Bcl-2 family, have been implicated in cell survival and death (34, 43). Downstream expression of these factors has been associated with the activation of various pathways including the NF-κB and Akt signal transduction pathways. Bax and Bcl-XL expression, a pro-apoptotic factor and anti-apoptotic factor, respectively, were found to decrease in C4-II cells at the 100 μg/ml ethanolic extract treatment. Previous studies have shown that NF-κB activation is associated with downstream activation of the anti-apoptotic factor, Bcl-XL (35). Therefore, the decrease in NF-κB activation in C4-II cells correlates with decreased expression of Bcl-XL and supports our observation that *M. oleifera* ethanolic extract promotes apoptosis in C4-II cells. Furthermore, the increased Akt levels exhibited in C4-II cells suggests that decreased Bax expression following treatment alone may be due to increased Akt activation, which has been shown to inhibit Bax activation (95).

Therefore, our results suggest that induction of C4-II apoptosis following treatment with the ethanolic extract may not be related to Bax, but dependent on the decreased expression of the anti-apoptotic factor, Bcl-XL.

What is interesting are the differences in expression between Bcl-XL and NF-κB in HeLa cells treated with the ethanolic extract. Following treatment alone, total NF-κB levels decreased, but Bcl-XL expression increased. These results suggest that another cell pathway than NF-κB may be promoting Bcl-XL expression in HeLa cells. Research suggests that Bcl-XL has an inhibitory effect on Bax and its role in apoptosis (34). While not significant (p=0.076), Bax expression was shown to decrease in treated HeLa cells. Therefore, our results suggest that the increase in HeLa cell killing exhibited by the ethanolic extract is not through apoptosis via Bax, but perhaps through another mechanism of cell death such as through the extrinsic apoptotic pathway or through autophagic cell death (88). For example, previous studies have shown that NF-κB inhibits autophagy in cells (91). Based on the decreased levels of NF-κB, the ethanolic extract may promote HeLa cell death via autophagy.

The effect of the *M. oleifera* ethanolic extract on antiviral pathways and the ability of the virus to replicate in cells was also examined. *M. oleifera* ethanolic extract decreased total STAT1 levels in IFN-treated and rwt infected C4-II and HeLa cells. This indicates that the ethanolic extract demonstrates an inhibitory effect on the type I IFN (antiviral) response (71). Therefore, the ethanolic extract may decrease activation of an antiviral response in infected cells to allow for efficient infection of rwt virus in C4-II and HeLa cells and thus promote cell killing. Interestingly, the ethanolic extract promoted phosphorylation of STAT1 in C4-II cells not treated with IFN which indicates that *M. oleifera* promoted STAT1 activation in treated cells. While most instances of STAT1 activation are associated with antiviral

responses, STAT1 also plays a role in cancer cells survival and growth (12). Following production of IFN, STAT1 has been shown to promote apoptosis in cancer cells through activation of caspases (84). Therefore, an increase in STAT1 activation cancer cells following treatment with the ethanolic extract may induce apoptosis of C4-II cells.

One concern for using *M. oleifera* in combination with VSV is the affect it may have on virus replication in cells. Our data suggests that *M. oleifera* ethanolic extract does not negatively impact virus replication in C4-II, HeLa, and SiHa cells. Furthermore, rM51R-M virus exhibited increased replication compared to rwt virus. This result is surprising given the nature of these virus strains. The rwt virus inhibits host gene expression and the type I IFN response to promote its own replication. In contrast, rM51R-M virus is defective at suppressing host gene expression (4). Therefore, rM51R-M virus should not be able to replicate as efficiently in infected cells as compared to rwt virus due to its ability to enhance the antiviral response. However, it is possible that in these cancer cells, rM51R-M virus stimulates additional factors to promote its own replication. Interestingly, the ethanolic extract was shown to enhance the replication of rM51R-M virus suggesting that *M. oleifera* may suppress activation of an antiviral response and allow for increased replication of rM51R-M in infected cells. To support this observation, we observed that the ethanolic extract decreased STAT1 levels VSV-infected C4-II and HeLa.

As previously mentioned, the methanolic extract demonstrated differences in its ability to kill cervical cancer cells as compared to the ethanolic extract. The methanolic extract did not induce cell death in cervical cancer cell lines. However, it stimulated STAT1 activation in IFN-treated cells. This suggests that the methanolic extract has the potential to promote an antiviral state. In contrast to the results from extract alone, the methanolic extract

decreased activation of an antiviral response in rM51R-M infected SiHa cells, which may promote infection and killing of SiHa cells by the mutant virus. These results are interesting when taking into account that the methanolic extract increased GFP expression in rM51R-M infected SiHa cells. In total, our results suggest that the methanolic extract promotes killing of SiHa cells by rM51R-M virus by inhibiting activation of the type I IFN response and promoting a permissive state in SiHa cells.

M. oleifera has demonstrated its ability to promote direct killing of cervical cancer cells by VSV, perhaps through modifications of cell signaling pathways associated with cell survival and proliferation and through modifications of the antiviral response. Furthermore, *M. oleifera* does not negatively impact the ability of VSV to replicate in cervical cancer cells. However, a critical aspect of oncolytic therapy is the ability of specific viruses or regimens to stimulate anti-tumor immunity, perhaps by promoting clearance of VSV-infected cervical cancer cells. In addition to indirect detection of virus-infected cancer cells, VSV has been shown to directly stimulate DC via binding of viral antigens to TLR7 (26). Following binding of VSV to DC, various events occur that will promote clearance of the virus infection. For example, increased expression of co-stimulatory receptors and increased production of pro-inflammatory cytokines set the stage for activation of other immune cells involved in virus clearance and activation of the type I IFN antiviral response (14). pDC secrete type I IFN, which protects nearby cells from future infection, and leads to activation of mDC (7). In addition to IFN, activated pDC also secrete pro-inflammatory cytokines such as IL-6 which can stimulate activation of NK cells to further promote clearance of virus-infected cells. Activation of mDC have also been shown to provide direct activation of T cells, which can lead to the development of adaptive immunity against subsequent infections

(7). It is interesting to point out the differences between rwt and rM51R-M viruses, and how they affect DC stimulation. As previously demonstrated, rwt virus suppresses stimulation of DC based on its ability to suppress host gene expression. In contrast, rM51R-M virus, which is defective at inhibiting host gene expression and the type I IFN response, stimulates DC subsets (7).

Our results found that the ethanolic extract stimulated pDC and mDC subsets and IL-6 production in rM51R-M infected cells. Therefore, the ethanolic extract has the potential to promote clearance of rM51R-M infected cancer cells by activating DC subsets. Furthermore, the increased production of IL-6 suggests that *M. oleifera* may stimulate additional immune cells. Our results demonstrated that NK and T cells were not activated by the addition of the ethanolic extract. However, the effect of the ethanolic extract on these cell populations may need to be measured at multiple time points post-infection. Because DC can stimulate NK cells, it is possible that the ethanolic extract may promote activation of these cells at an earlier time point. For example, various experiments designed to examine NK activation are based on a 4 hour incubation (24). Furthermore, subsequent incubation time may be needed to determine if *M. oleifera* influences T cell activation. While 20 hours is sufficient time for stimulation of these cells, some studies have shown that peak expression of CD69, indicative of T cell activation, can be detected at 48 hours post treatment (83).

When comparing the ethanolic and methanolic extracts and their influence on cytokine production in PBMC populations, it is interesting to note the similarities and differences between the extracts and their effect on LPS treated and virus-infected cells. Both extracts were shown to increase IL-6 levels and further stimulate DC in LPS-treated PBMCs. Previous studies have shown that *M. oleifera* demonstrates anti-inflammatory properties (16).

Although an increase in pro-inflammatory cytokine expression was not hypothesized, these results may identify the role of *M. oleifera* in protection against bacterial infections through stimulation of DCs (74). Interactions between LPS and TLR4 on DCs promote maturation of these cells and an inflammatory response (32). These results, in combination with our observations in rM51R-M virus-infected PBMC, suggest that while *M. oleifera* is capable of inhibiting pro-inflammatory signaling, the extracts examined also promote inflammatory responses to infections. These results support the role of *M. oleifera* in oncolytic therapies, and demonstrate that *M. oleifera* has the potential to promote clearance of virus-infected cancer cells and stimulate anti-tumor immunity.

A main goal of our study was to determine how immune cells react in the context of oncolytic virus and *M. oleifera* treatment. Our results indicated that the *M. oleifera* ethanolic extract, while not significant (p=0.055), promoted IL-6 production in rwt-infected HeLa-PBMC co-cultures, whereas individually infected PBMC and HeLa cell populations exhibited decreased IL-6 production. Increased IL-6 production may be beneficial following infections by rwt virus. Previously, we have seen that rwt suppresses infection of a pro-inflammatory response (4). Therefore, increased activation of an immune response may promote detection of rwt-infected cells by key immune cells, such as DC, to promote clearance of these cells. Therefore, rwt virus used in conjunction with *M. oleifera* may serve as a safer alternative compared to using rwt alone for treating cervical cancer cells. Based on previous studies, defects in the rM51R-M virus allow for activation of an antiviral response (5). Although IL-6 and TNF levels were much higher in rM51R-M infected HeLa-PBMC co-cultures, the ethanolic extract did not significantly alter cytokine production in these co-cultures. It is interesting to note that of the experiments performed, both increases and

decreases in IL-6 and TNF expression were observed. This suggests that *M. oleifera* promotes different responses in individuals, but does not significantly alter the ability of immune cells to induce a response against rM51R-M virus infection. Further studies are necessary to determine if the responses exhibited in our co-culture studies are similar in other cervical cancer cell types.

It is important to ask whether the degree of immune stimulation induced by *M. oleifera* is significant in the context of cancer therapies. Although *M. oleifera* significantly altered cytokine levels and immune cell stimulation in our experimental conditions, these changes may not be significant in the context of a tumor environment. Variations in cytokine levels and DC stimulation induced by *M. oleifera* may be too minute to significantly alter the tumor microenvironment. Therefore, future studies are needed to further examine whether *M. oleifera* will have a significant effect on the tumor microenvironment.

Our research suggests that *M. oleifera* has the potential to promote killing of cervical cancer cells both alone and in combination with VSV by altering cell signaling pathways that promote an anti-proliferative and anticancer environment. Furthermore, results show that a 6 hour pre-incubation with the ethanolic extract inhibits activation of the type I IFN antiviral response which may allow for increased VSV infection and replication in cancer cells. Infection by VSV following pretreatment with the *M. oleifera* promotes activation of DCs for clearance of VSV-infected cancer cells. Future studies will examine immune cell stimulation with co-culture studies to better understand how *M. oleifera* alters the tumor microenvironment. Additionally, the influence of *M. oleifera* on cytokine production and

immune cell stimulation in the tumor microenvironment via mice models will provide an in-depth understanding of how *M. oleifera* may serve to promote the oncolytic activity of VSV in cervical cancer cells.

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Vita

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