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Research Article

Nigrospora sphaerica Products from the Flowering Dogwood Exhibit Antitumorigenic Effects via down-regulation of the Translational Regulator, Ribosomal S6 Protein

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Abstract: The utility of diverse species of endophytic fungi as a viable source for drug agents with clinical applicability for the treatment of human diseases continues to expand. In this study, we examined secondary metabolites of *Nigrospora sphaerica* isolated from the flowering dogwood, *Cornus florida* L., for their anticancer properties on lung cancer and glioblastoma. Molecular identification of *N. sphaerica* was determined using internal transcribed spacer rDNA sequence. The expression of translational pathway proteins was examined after exposure to various crude extract concentrations (2 µg/ml, 4 µg/ml, and 8 µg/ml) using immunoblotting procedures, while tumor cell migration analysis was performed using Boyden chamber assays. Crude *N. sphaerica* extracts exhibited antiproliferative and antimigratory effects on solid tumors as determined by cell proliferation and cell migration assays, respectively. The antitumorigenic effects of *N. sphaerica* were as a consequence of negatively regulating the PI3K/Akt/mTOR translational control signaling pathway, a canonical mechanistic axis that contributes to the maintenance and progression of several human cancers. To the best of our knowledge, this is the first evidence that demonstrates *N. sphaerica* from *C. florida* inhibits tumor cell migration and thus disease recurrence a major factor in the therapeutic resistance of cancers to chemotherapeutic agents.

Keywords: *natural product; fungi; secondary metabolite; intracellular signaling; cancer*

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0 Background

Endophytic fungi are organisms that colonize the internal tissues of plants without damaging the host[1] and have proven to be a robust source for the discovery of novel secondary metabolites that exhibit a wide range of biological and biomedical applications[2-4]. A candidate source and potential host of endophytic fungi with biomedical applications are the flowering dogwood, *Cornus florida* L., which has been shown to produce antiparasitic compounds for the treatment of malaria[5]. Germane to the present study *C. florida* has also been shown by Vareed *et al*.[6] to produce the phenolic pigment anthocyanin, which displayed antitumor activity in colon, breast, lung, stomach, and central nervous system cancers. To this end, the current study examined the antitumor activity of crude extracts prepared from the endophytic fungus *Nigrospora sphaerica* isolated from *C. florida*, which has previously been described as an endophyte resource for several biomedical applicable compounds such as antibiotics[7,8], antiviral anthraquinones, azaphilones[9], and the antifungal griseofulvin[10]. Of specific significance, the alkaloid chemotherapeutic drug, vinblastine, is also a product of the endophytic fungus *N. sphaerica* although isolated from *Catharanthus roseus* (Madagascar periwinkle)[11]. However, the clinical caveat with the utility of the microtubule poison vinblastine for the treatment of human cancers has been its associated toxicities, making it imperative to identify sources for chemotherapeutic agents with improved toxicity profiles. Here, we examined the antiproliferative effect of crude extracts from

N. sphaerica on lung and glioblastoma cancer cell lines, as well as the ability of *N. sphaerica* extracts to inhibit cancer cell migration. The results of this study provide experimental evidence that the endophytic fungus *N. sphaerica* in flowering dogwoods is a promising source of bioactive metabolites with potential antitumor and antimetastasis activities.

1 Methods

2.1 Sample collection and fungal isolation

Healthy stem samples were collected from mature *C. florida* plants at Otis L. Floyd Nursery Research Center (Tennessee State University, McMinnville, TN). The samples were placed in sterile bags, stored at 4°C, and processed within 24 h of sampling. Endophytic fungi were isolated using methods as previously described by Schulz *et al*.,[12] with some modification. Stem samples were cleaned thoroughly under running tap water and cut into 1.0–1.5 cm pieces. Samples were the next surface sterilized with 70% ethanol for 1 min and 10% sodium hypochlorite for 3–5 min. Samples were subsequently rinsed 3 times with sterile distilled water, blotted on paper towel, and allowed to air dry. Next five segments of vascular tissue (3–4 mm) were transferred onto Petri dishes containing acidified potato dextrose agar (PDA), incubated at room temperature, and observed for the appearance of mycelial growth. Pure fungus was obtained by at least two or three successive subculturing on PDA (PDA; Sigma-Aldrich, St. Louis, MO).

2.2 Molecular identification and phylogenetic analysis

Fungal genomic DNA was extracted using the FastDNA kit (MP Biomedicals, Santa Ana, CA) per manufacturers' instructions. Next polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) region was performed using the universal primers ITS1 and ITS4[13]. Subsequently, the PCR product was purified using Exosap (USB-Affymetrix, Santa Clara, CA) and sequenced by Eurofins Genomics (Louisville, KY). Next, the ITS sequence was analyzed using BLASTN (GenBank National Centre of Biotechnology Information-NCBI; https://www.ncbi.nlm.nih.gov), and the identity of the organism was determined based on the closest match in the GenBank database. DNA sequence similarities of ≥99–100% homology were used to identify the fungus *N. sphaerica* studied here. Subsequently, the *N. sphaerica* ITS sequence and reference sequences retrieved from GenBank were aligned using ClustalW before phylogenetic tree construction in MEGA7 software[14]. The neighbor-joining (NJ) method was used to infer evolutionary history[15]; the p-distance method was used to compute evolutionary distances[16], and bootstrap analysis with 1000 replication was used to assess the robustness of the phylogenetic tree[17].

2.3 Crude extract preparation

For crude extract preparation 5–7, 6 mm^2 agar plugs of fungal culture were inoculated in 1000 ml Erlenmeyer flasks containing 300 ml of potato dextrose broth (Sigma-Aldrich, St. Louis, MO, USA). Flask was then incubated at 28°C on a rotary shaker at 160 rpm for 10 days. After incubation, the fermented medium was centrifuged at 4000 rpm to separate mycelia from the supernatant. Subsequently, supernatant was filter sterilized (0.22 µm) and extracted 3 times with equal volumes of ethyl acetate (Fisher Scientific, Fair Lawn, NJ, USA) using separatory funnel. Next, the organic phase was removed using rotary evaporator under vacuum and resultant crude extract was lyophilized. The stock solution of 2.0 mg/ml crude extract was prepared in dimethyl sulfoxide (DMSO) (DMSO; Amresco Solon, OH, USA) and stored at −20°C for further analysis.

2.4 Cells culture conditions and reagents

U251 human glioblastoma cells were purchased from Sigma-Aldrich (St. Louis, MO, USA) and A549 human lung carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 100 nM MEM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), and penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO_2.

2.5 Crystal violet cell proliferation assay

U251 and A549 cells were plated in 24-well plates and treated with 2 µg/ml, 4 µg/ml, 8 µg/ml, and 10 µg/ml of fungal crude extract, while vehicle-treated control cells were treated with DMSO. For dose-response experiments, cells were treated with *N.*

sphaerica (2 µg/ml, 4 µg/ml, 8 µg/ml, and 10 µg/ml) fungal extract or vehicle and allowed to incubate for 48 h, while for time-course experiment, cells were plated, treated as explained above, and incubated for 2, 4, and 6 days. At the end of each time point, the tissue culture medium was removed; the cell monolayer was fixed with 100% methanol for 5 min and stained with 0.5% crystal violet in 25% methanol for 10 min. Cells were then washed 3 times 5 min each with distilled water to remove excess dye and allowed to dry overnight at room temperature. The incorporated dye was then solubilized in 0.1 M sodium citrate (Sigma-Aldrich, St. Louis, MO, USA) in 50% ethanol. Next, 100 µl of experimental samples were transferred to 96-well plates and optical densities were recorded at 595 nm using an X-mark microplate absorbance spectrophotometer (BioRad, Hercules, CA, USA).

2.6 Cell migration

Motility assays were conducted according to manufacturer's instructions (CellBiolabs Inc., San Diego, CA, USA). Cell suspensions containing 0.5–1.0 × 10^6 cells/ml were treated with DMSO (vehicle control) or 2 µg/ml of crude extract in serum-free media, while 500 µl of media containing 10% fetal bovine serum was added to the lower chamber of the migration plate. Next, 300 µl of cell suspension containing vehicle control or 2 µg/ml of crude extract were then added to the inside of each insert and allowed to incubate for 24 h at 37°C and 5% CO_2. Subsequently, non-migratory cells were removed from plate inserts (per manufacturer's instructions), while migratory cells were stained with crystal violet and the dye solubilized (per manufacturer's instructions). Optical densities were read at 595 nm using an X-mark microplate absorbance spectrophotometer (BioRad, Hercules, CA, USA).

2.7 Caspase 3 activity

To assess caspase 3 activity, cells were plated in serum-free DMEM for 24 h, treated with 2 µg/ml, 4 µg/ml, and 8 µg/ml crude extract or vehicle (DMSO), and allowed to incubate for 24 h. After incubation, cells were rinsed with cold phosphate buffer saline (PBS) (PBS; Bio-Rad Laboratories, Hercules, CA, USA) and lysed in CelLytic M cell lysis reagent (Sigma-Aldrich, St Louis, MO, USA). Protein concentrations were then determined using the Bradford method. Next, caspase 3 activity was determined using the CaspACE Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.8 Western blotting

For western blot experiments, cell wells were treated with 2 µg/ml, 4 µg/ml, and 8 µg/ml crude extract or vehicle (DMSO) for 3 h, rinsed with PBS, and lysed with CelLytic M cell lysis reagent (Sigma-Aldrich, St Louis, MO, USA). Protein concentrations were subsequently determined using the Bradford method. Proteins were next run in 4–15% sodium dodecyl sulfate-polyacrylamide gels (BioRad, Hercules, CA, USA) and transferred to nitrocellulose membranes. Nitrocellulose membranes were then incubated overnight at 4°C with primary antibodies to detect the protein expression of pAkt, pS6 ribosomal protein, p90 RSK, 4EBP1, GAPDH, and β-actin (Cell Signaling Technology, Danvers, MA, USA). Nitrocellulose membranes were next washed 3 times 5 min each in PBS containing 0.05% Tween 20, incubated with an horseradish peroxidase-conjugated secondary antibody for 3 h at room temperature and washed. Proteins were visualized and analyzed by enhanced chemiluminescence (Thermo Scientific, Nashville, TN, USA) and a UVP BioSpectrum imaging system (UVP, Upland, CA, USA).

2.9 Statistical analysis

Cell proliferation, migration, and time-course experiments were each performed at least 3 times in duplicate. Means were determined by averaging duplicate samples within each independent experiment. Student's *t*-tests and ANOVA with Sidak's *post hoc* statistical analysis were used to evaluate significance.

3. Results

The endophyte *N. sphaerica* has been previously reported in a number of plant species[11,18-20]. To the best of our knowledge, this study is the first report to show the association of *N. sphaerica* with *C. florida* as confirmed using ITS rDNA sequence and phylogenetic analysis [Figure 1].

In addition, few studies have evaluated the anticancer properties of secondary metabolites from *N. sphaerica.* Initial experimental studies on the effect of the endophytic fungus *N. sphaerica* on human cancer cells were determined through dose-response analysis on lung cancer and glioblastoma cell lines, which displayed

a concentration-dependent effect [Figures 2 and 3]. Glioblastoma cells treated with 2 µg/ml of *N. sphaerica* crude extract showed a 25% decrease in cell viability and an 84% decrease in cell viability when treated with 10 µg/ml, while lung cancer cells treated with these concentrations displayed a 17% and 62% decrease, respectively, as compared to vehicle-treated control cells [Figure 2]. The quantitative effect of this *Nigrospora* fungus on lung and glioblastoma cancer cell viability was further supported by ANOVA analysis which showed a statistically significant difference ($P < 0.05$) across all concentrations examined. Subsequently, time-course analysis was performed on lung and glioblastoma cancer cells exposed to crude extracts of *N. sphaerica.*

Consistent with dose-response experiments cells treated with *N. sphaerica* crude extracts ranging in concentration between 2 µg/ml and 10 µg/ml also displayed concentration-dependent effects of crude extracts on glioblastoma and lung cancer cells, with the highest doses having the most pronounced effect on decreasing cell viability [Figure 4]. This is supported by a 93% and 96% decrease in viable lung cancer and glioblastoma cells, respectively, observed 6-day post-exposure with 10 µg/ml of *N. sphaerica* crude extract, as compared to vehicle-treated control cells examined at this same time point [Figure 4]. In addition, treatment with 10 µg/ml of *N. sphaerica* induced a cytostatic cellular response in lung cancer cells and a cytotoxic cellular response in glioblastoma cells over the 6-day time period [Figure 4]. These data are consistent with the antiproliferative effects of *N. sphaerica* products observed in leukemia and breast cancer cells[11,21].

Figure 4 shows an experiment representative of three independent experiments performed in duplicate (means ± SE) that displayed similar results.

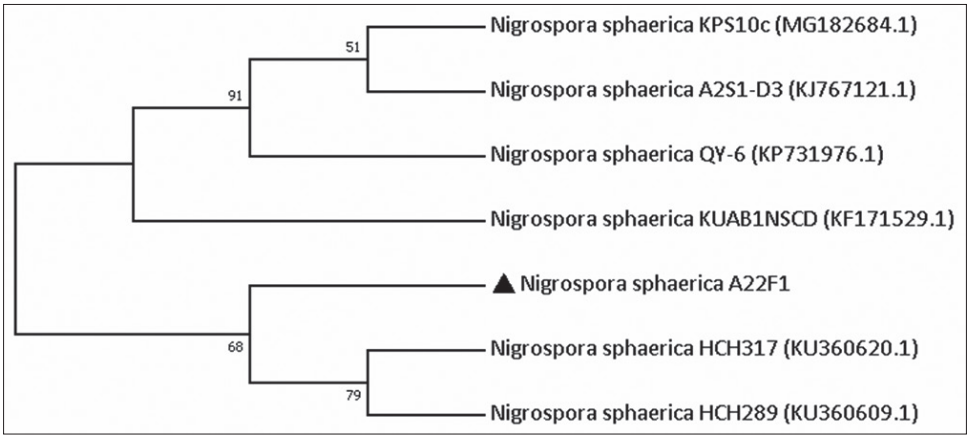

Figure 1. Neighbor-joining phylogenetic tree generated by p-distance method based on ITS sequence of endophytic *Nigrospora sphaerica* (▲) isolated from *Cornus florida*. The optimal tree with the sum of branch length = 0.02020992 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

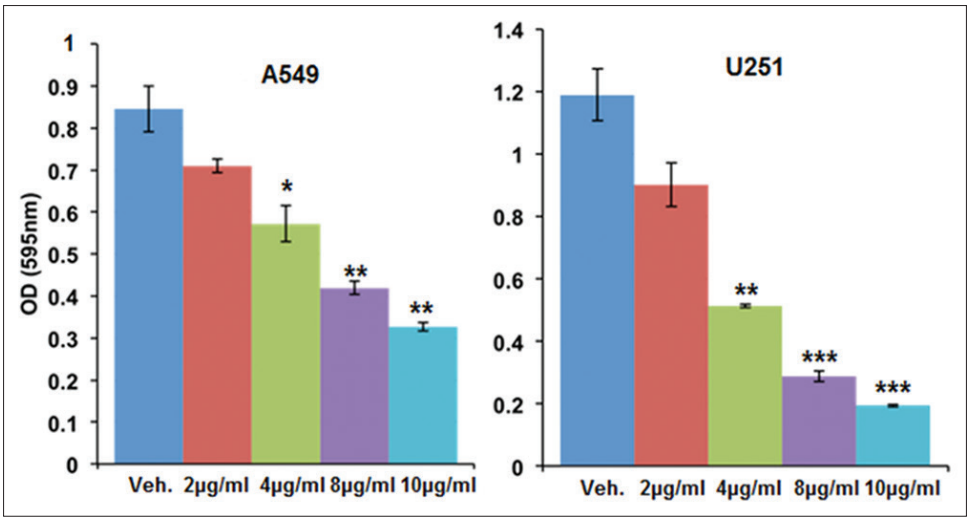

Figure 2. Dose-response effects of *Nigrospora sphaerica* on lung cancer (A549) and glioblastoma cells (U251). Cells treated with 2–10 µg/ml of *N. sphaerica* crude extract caused a reduction in cell viability. Data shown are representative of at least four experiments performed in duplicate (means ± SE) that showed similar results (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$ compared to vehicle-treated control cells)

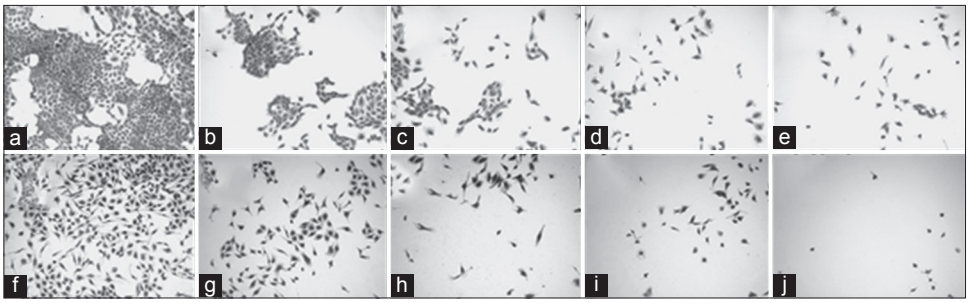

Figure 3. Qualitative dose-response assessment of *Nigrospora sphaerica* crude extract on lung cancer (a-e) and glioblastoma cells (f-j). Concentrations of *N. sphaerica* crude extract: Dimethyl sulfoxide vehicle control (a, f); 2 µg/ml (b, g); 4 µg/ml (c, h); 8 µg/ml (d, i); 10 µg/ml (e, j). Figure 3 shows a single experiment representative of four independent experiments that displayed similar results

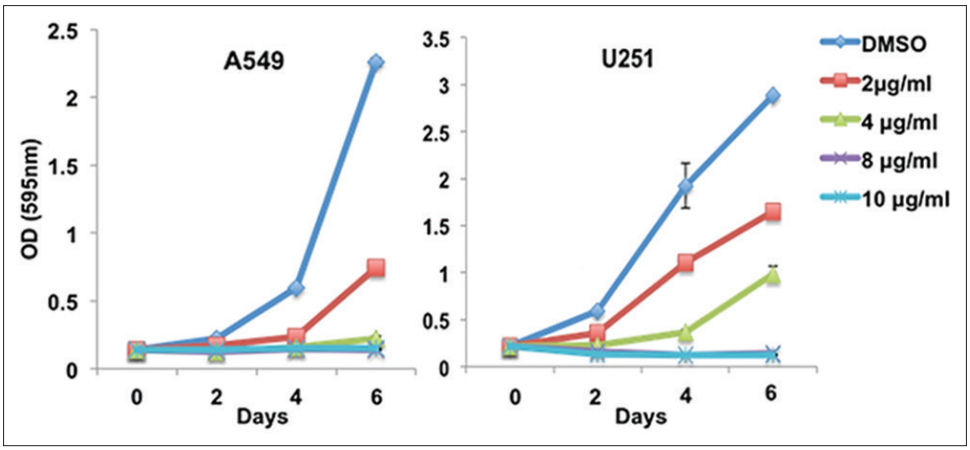

Figure 4. Time-course analysis of *Nigrospora sphaerica* crude extract on lung cancer (A549) and glioblastoma cells (U251). *N. sphaerica* crude extract (10 µg/ml) induced a cytotoxic and cytostatic cellular response as compared to vehicle-treated control cells.

To gain insight into the role that apoptotic cell death played in the reductions in cancer cell viability as a consequence of exposure to *N. sphaerica* crude extracts, caspase 3, a known executioner of apoptosis, was evaluated in lung cancer and glioblastoma cells [Figure 5] post-exposure to crude extracts. Our data showed a diminutive increase in caspase 3 activity in both cell lines treated with *N. sphaerica* crude extracts [Figure 5]. In addition, we assessed the alternative cell death mechanism autophagy, as a means that underlie reduced cell viability of lung and glioblastoma cells treated with *N. sphaerica* crude extract. Immunoblotting data showed no change in the autophagy marker LC3 in response to *N. sphaerica* crude extract exposure (data not shown). We further analyzed the expression of proteins that regulate translational control (pAkt, pS6 ribosomal protein, and 4EBP1) and ERK (p90RSK) signaling pathways as mechanistic contributors of the antiproliferative effect of *N. sphaerica* crude extract on solid tumor cell lines studied here. *N. sphaerica* crude extract exposure caused a demonstrative decrease in the expression of the translational regulator pS6 ribosomal protein in lung cancer cells [Figure 6] but no measurable change in the ERK signaling protein p90RSK. In contrast, *N. sphaerica* crude extract had no effect on translational control and ERK signaling proteins evaluated herein glioblastoma cells (data not shown).

Since tumor recurrence is a manifestation of the resistance of many cancers to clinically used chemotherapeutic agents, we next examined the efficacy of *N. sphaerica* crude extracts to inhibit lung cancer and glioblastoma cell migration. Boyden chamber assays revealed that cells treated with 2 µg/ml of *N. sphaerica* crude extracts decreased ($P < 0.05$) tumor cell migration as compared to vehicle-treated control cells [Figure 7], paralleling recent findings by Mady *et al*.[22] that showed the endophytic fungus *Penicillium chrysogenum* impaired breast cancer cell migration.

4 Discussion

We have shown here that metabolic products of the endophytic fungus *N. sphaerica* have antitumorigenic

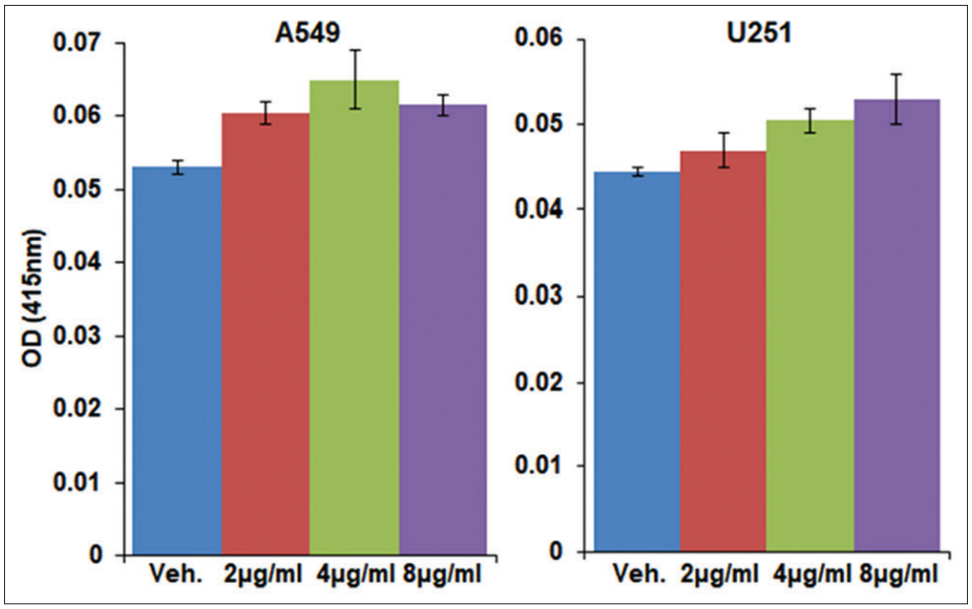

Figure 5. Evaluation of apoptosis in response to *Nigrospora sphaerica* crude extract. Treatment of lung cancer (A549) and glioblastoma (U251) cells with *N. sphaerica* crude extract induced a slight increase in caspase 3 activity. Caspase 3 activity data shown are representative of at least three experiments performed in duplicate (means ± SE) that showed equivalent results, while immunoblots presented are representative of at least three experiments that showed similar results

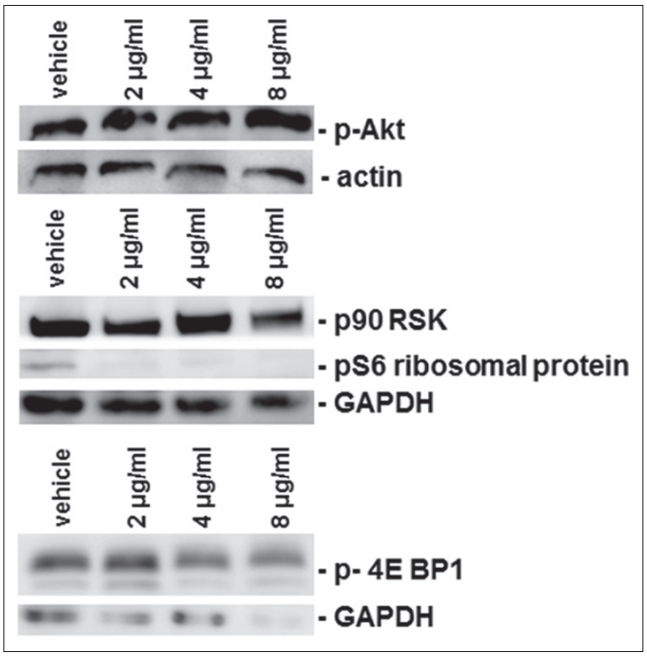

Figure 6. Western blot analysis of translational control and ERK1/2 signaling mediators in lung cancer cells. *Nigrospora sphaerica* crude extract promoted a downregulation of pS6 ribosomal protein in A549 lung cancer cells. Immunoblots presented are representative of at least four experiments that showed equivalent results. GAPDH and actin were used as loading controls to assess that lanes were loaded with the same amount of total proteins

properties as exhibited by the antiproliferative effects of *N. sphaerica* crude extracts from *C. florida* on lung cancer and glioblastoma cells. Our findings are consistent with previous experimental studies that have also shown secondary metabolites produced by *N. sphaerica* isolated from *C. roseus and Asteraceae* inhibited the proliferation of breast cancer and leukemia cells, respectively. More specifically, Ayob *et al*.[11] demonstrated that the vinca alkaloid, vinblastine produced by *N. sphaerica* from *C. roseus* inhibited breast cancer cell growth. It should be mentioned that the concentration, however, of vinblastine used to inhibit breast cancer cells by Ayob *et al*.[11] was 3 times that of the highest concentration of *N. sphaerica* crude extract used in this study to inhibit the growth of solid cancer cell lines. This suggests that cancer cells may be more sensitive to *N. sphaerica* products extracted from *C. florida* and that endophytic fungi products from this plant are more effective at inhibiting cancer cell proliferation. In addition, Gallo *et al*.[21] showed that diterpene aphidicolin, a *N. sphaerica* product of Asteraceae, had an antiproliferative effect on leukemia cells that were attributed in part to G2/M cell cycle arrest, apoptosis, and autophagy. In contrast, *N. sphaerica* crude extracts in our study did not induce apoptotic cell death or an autophagic response, but instead promoted a decrease in the translational regulator, pS6 ribosomal protein an effector molecule in the PI3K/Akt/mTOR signaling pathway regulated by mTORC1 (Raptor-mTOR) a known regulator of cell growth, glucose and lipid metabolism, autophagy, and protein synthesis[23]. Although the change in pS6 ribosomal protein expression provides a mechanistic basis for the antiproliferative effects of

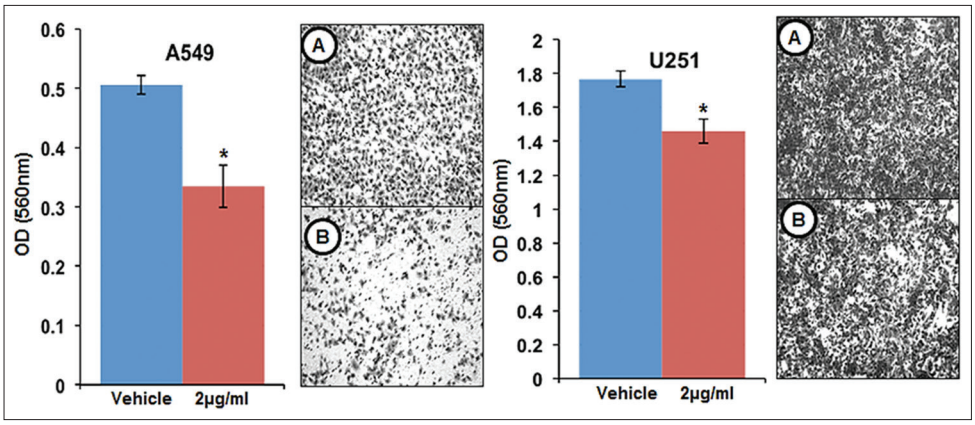

Figure 7. *Nigrospora sphaerica* crude extract impairs solid tumor cell migration. Lung cancer (A549; left panel) and glioblastoma cell (U251; right panel) migration were significantly diminished after treatment with 2 µg/ml of *N. sphaerica* crude extract. Data shown are representative of three independent experiments performed in duplicate (means ± SE) showing similar results (*$P < 0.05$ compared to vehicle-treated control cells). Vehicle-treated control cells (blue bars); cells treated with 2 µg/ml of *N. sphaerica* crude extract for 24 h (red bars)

lung cancer and glioblastoma cells seen in response to *N. sphaerica* crude extracts, the downregulation of this translational regulatory protein has also been associated with impeding cancer cell migration. In a study by Kim *et al*.,[24] it was demonstrated that genetic downregulation of pS6 ribosomal protein inhibited esophageal cancer cell migration and invasion. This was further supported in studies by Chen *et al*.,[25] who observed this same phenomenon in non-small cell lung cancer. Taken together these investigations support the notion that impairment of lung cancer and glioblastoma cell migration to *N. sphaerica* crude extracts isolated from *C. florida* is also a consequence of pS6 ribosomal protein reduction.

5 Conclusion

The antimigratory effects in conjunction with the antiproliferative properties of *N. sphaerica* crude extracts from *C. florida* on solid tumor cell lines observed in our study provide evidence that endophytic fungi isolated from this plant are able to impede the continued development and recurrence of human cancers. Furthermore, findings here expand the pool of botanical resources that can be used to evaluate secondary metabolites from endophytic fungi for their anticancer properties to include the flowering dogwood, *C. florida*.

6 Acknowledgments

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The authors declare that they have no competing interests.

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