Lippia origanoides extract induces cell cycle arrest and apoptosis and suppresses NF-κB signaling in triple-negative breast cancer cells

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Abstract. Treatments targeting hormone receptors typically fail to provide a positive clinical outcome against triple-negative breast cancers (TNBC), which lack expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2/neu). Towards identifying viable treatments for aggressive breast cancer, we have tested an extract of the tropical plant *Lippia origanoides* (LOE) on TNBC and normal cells lines to uncover its potential anticancer effects. Treatment with LOE reduced TNBC cell viability in a dose-dependent manner to a greater extent than in normal mammary epithelial MCF10A cells. In MDA-MB-231 cells, LOE was found to halt the cell cycle in the G0/G1 phase via cyclin D1 and cIAP2 regulation, and induce apoptosis without promoting necrosis via caspase-8/-3 and PARP cleavage. Constitutive nuclear factor-κB (NF-κB) signaling has been shown to contribute to the heightened inflammatory state and survival in TNBC cells. Herein, we also provide evidence that LOE inhibits NF-κB signaling by reducing RIP1 protein levels in MDA-MB-231 cells. These studies reveal that LOE suppresses key features of the progression of aggressive breast cancer cells and provides a basis for further definition of its underlying mechanisms of action and anticancer potential.

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Abbreviations: cIAP, cellular inhibitor of apoptosis; ER, estrogen receptor; Her2/neu, human epidermal growth factor receptor 2; LOE, *Lippia origanoides* extract; NF-κB, nuclear factor κB; PARP, poly-ADP ribose polymerase; PR, progesterone receptor; TNBC, triple-negative breast cancer

Key words: triple-negative breast cancer, *Lippia*, apoptosis, phytochemicals, NF-κB

Introduction

Breast cancer is a major health concern worldwide, and a leading cause of cancer-related deaths among women in the United States where over 245,000 new cases are diagnosed and 45,000 deaths occur each year (1). In the majority of diagnoses, this disease presents as a hormone-sensitive breast cancer that overexpressed estrogen receptor (ER), progesterone receptor (PR) and/or human epidermal growth factor receptor 2 (HER2/neu). These cancers typically depend on hormone-receptor signaling for tumor growth and progression. Consequently, conventional therapy against the majority of breast cancers targets these receptors (e.g., tamoxifen, a selective estrogen receptor modulator and herceptin, an antibody that inhibits HER) or attempts to inhibit enzymes involved in estrogen synthesis (e.g., aromatase inhibitors such as letrozole) (2-4). In contrast, the most deadly subtype of this disease is known clinically as triple-negative breast cancer (TNBC), and constitutes approximately 15% of invasive breast cancers. TNBCs lack expression of genes coding for ER, PR and HER2/neu, are not dependent on hormonal signaling for progression, and do not respond to conventional therapy (5).

TNBCs are frequently of the highly invasive and recurrent basal-like subtype (6). They are also associated with a variety of aberrations (e.g., higher incidence of TP53 mutations and the constitutive activation of pro-survival and inflammatory pathways such as MAPK and NF-κB), all of which may contribute to their growth, survival and resistance to chemotherapy (7-10). NF-κB signaling maintains an autocrine loop of pro-inflammatory cytokines such as IL-6 and TNF-α, promotes expression of pro-survival factors such as Bcl-xL, cIAP1 and cIAP2 and proteins involved in cell cycle progression (e.g., cyclin D1), as well as invasion and metastasis markers such as MMP-9 and vimentin (11-17). Constitutive activation of NF-κB in triple-negative breast cancer may contribute to its aggressiveness and ability to maintain hormone-independent growth. Inhibition of NF-κB signaling is thus a viable target for novel therapeutics against TNBC.

Plant-derived molecules have been a critical source of cancer therapeutic agents. Generally, there are four main classes of plant-derived anticancer drugs currently in clinical use: *Vinca* alkaloids, taxanes, epipodophyllotoxins and camptothecins; the former two have entered clinical use against breast cancer following successful trials (18,19). Studies have also identified other natural plant compounds as capable of exerting pleiotropic anticancer effects. Plumbagin, for example, was shown to inhibit the DNA-binding activity of NF-κB and induce apoptosis in triple-negative MDA-MB-231 breast cancer cells (20). Further, halofuginone inhibited the nuclear localization of NF-κB and AP1, which are critical transcriptional activators of matrix metalloproteinase-9 (MMP-9), thereby reducing migration and invasiveness of these cells (21). Curcumin, honokiol, resveratrol and pinocembrin, among others, have also been shown to possess potent anti-cancer effects (22-25). These positive outcomes highlight the importance of fully uncovering the untapped clinical potential of natural compounds.

Herein, we report on an extract from *Lippia origanoides*, a medicinal tropical plant native to South America for its potential anticancer effects on TNBC. Also known as 'oregano de monte' or mountain oregano, infusions of the plant have been used traditionally to alleviate gastrointestinal ailments and as topical analgesics (26). Towards a better understanding of *L. origanoides* actions and health benefits, the novel *L. origanoides* extract (LOE) used in this study was originally isolated by supercritical fluid extraction and characterized using HPLC-MS (27,28). The LOE was found to have numerous major components including pinocembrin, carvacrol, thymol and trans-β-caryophyllene. Studies using commercially available reagents of these compounds have shown they have pro-apoptotic and anti-proliferative effects on various cancer cell lines. For example, pinocembrin, a flavanone which comprises nearly 55% of the total LOE, has been shown to decrease viability and prevent epithelial-mesenchymal transition induced by TGF-β in Y-79 retinoblastoma cells (25). Further, trans-β-caryophyllene and α-humulene, two sesquiterpenes present in the extract, were shown to synergize and inhibit cell growth and proliferation in MCF-7 breast cancer cells (29). Strikingly, several components of LOE have shown inhibitory effects on NF-κB signaling, a key survival and proliferative pathway in TNBC (30-34). These reports support the idea that *L. origanoides* may be a source of novel components that can effectively target aggressive breast cancer.

Our results show that treatment with LOE leads to a G0/G1 phase halt and apoptosis in MDA-MB-231 triple-negative breast cancer cells without promoting necrotic cell death. Further, we reveal that cell cycle proteins and apoptotic markers, as well as key NF-κB regulatory molecules, are modulated by treatment with LOE, thereby shedding light on a mechanism of action behind the anticancer effects of LOE. These data provide an important first step towards defining the potential utility of LOE in the identification and development of novel therapeutic strategies for TNBC.

Materials and methods

Plant material and extract. Lippia origanoides plants were collected from the Chicamocha River Canyon (Los Santos, Santander, Colombia). Taxonomic identification of *L. origanoides* was performed by Dr José Luis Fernández Alonso (National University, Bogotá, Colombia). The *L. origanoides* specimen (COL560259) was placed in the Colombian National Herbarium (Bogotá). Fresh leaves and flowers from *L. origanoides* were used for extraction as previously described (28). The extract was dissolved in methanol at a concentration of 50 mg/ml (stock solution), and then different extract concentrations were prepared in methanol for *in vitro* bioassays.

Cell culture. Triple-negative breast cancer (MDA-MB-231 and CRL-2321) and normal mammary epithelial (MCF10A) cell lines were obtained from the American Type Culture Collection. MCF10A-H cells, derived via H-*ras* transformation of MCF10A cells, were a kind gift from Dr Barbara Stefanska, Purdue University. MDA-MB-231 cells were cultured in Dublecco's modified essential medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies). MCF10A and MCF10A-H cells were cultured in a 1:1 ratio of DMEM:Ham's F12 supplemented with 5% horse serum (HS; Atlanta Biologicals), 20 ng/ml human epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 10 μg/ml bovine insulin (Sigma-Aldrich), 100 IU/ml penicillin and 100 μg/ml streptomycin. CRL2321 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Mediatech Inc., Herndon, VA, USA) supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Assessment of metabolic activity via MTT assay. MCF10A, MCF10A-H, MDA-MB-231 and CRL-2321 cells were seeded at 10^5 cells/well in 96-well plates and allowed to attach overnight. Spent media was then replaced with fresh media containing treatment dosages between 0.15-0.2 mg/ml LOE. Media in control wells was replaced with fresh media without additives (No treatment, NT) or media containing the vehicle, methanol (Veh). Following 24 h treatment, 12 mM of MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Life Technologies), was added to the cells followed by incubation for 4 h at 37˚C. Formazan crystals formed at the end of incubation period were dissolved using dimethyl sulfoxide (DMSO) and plates were read at 570 nm with a reference wavelength of 630 nm.

Assessment of cell cycle arrest via flow cytometry. For cell cycle assay, MDA-MB-231 were seeded in 6-well plates at a concentration of $6x10^5$ cells/well. Cells were allowed to attach overnight followed by replacement of spent media with serum-free media after 24 h to allow for synchronization of cell cycle. Cells were then treated in fresh media containing LOE at 0.06 and 0.15 mg/ml. Controls were treated in triplicate with either serum-free media as a positive control or media containing the vehicle, methanol. At the end of 36 h of treatment, media was aspirated, cells were washed once with 1X Phosphate Buffered Saline (PBS) and collected via trypsinization. Samples were centrifuged at 3,000 rpm

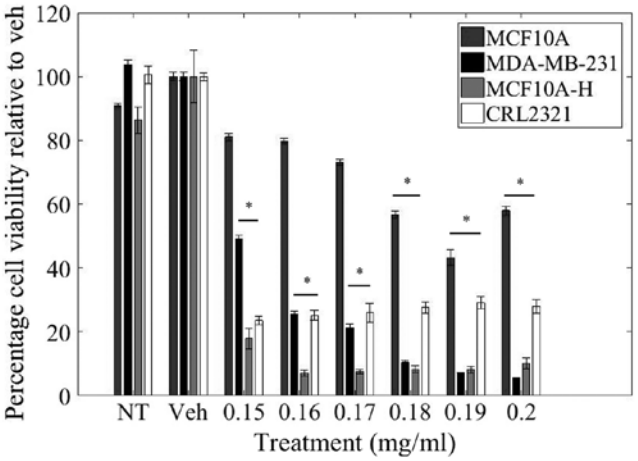

Figure 1. *Lippia origanoides* extract (LOE) impacts the viability of triple-negative breast cancer cells to a greater extent than normal-like cells. MDA-MB-231, MCF10A-H, CRL2321 and MCF10A cells were seeded in 96-well plates and treated with indicated concentrations of LOE, Methanol (Veh) or left untreated (NT) for 24 h and subjected to MTT assay. This was followed by absorbance reading at 570 nm. n=10 replicates from 2 separate experiments; *P<0.0001, significantly different from vehicle-treated cells.

for 5 min and supernatant was drained. Cell pellets were washed and resuspended in PBS containing 2.5 mM EDTA (1X PBS-EDTA buffer). Cells were then fixed by adding cell suspension drop-wise into microcentrifuge tubes containing 1 ml ice-cold 70% ethanol. Fixed cells were stored at -20˚C until staining. For staining, fixed samples were centrifuged at 4000 rpm for 5 min and supernatant was drained following which cells were washed and resuspended in 200 μl 1X PBS-EDTA buffer. Samples were centrifuged at 4000 rpm for 5 min and supernatant was drained. Next, 200 μl Muse® Cell Cycle Staining Reagent (EMD Millipore, Billerica, MA, USA) containing propidium iodide and RNase A was added to each sample. Samples were stored at room temperature for at least 30 min to allow for adequate staining before being run through an FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) at the Bindley Bioscience Center, Purdue University.

Assessment of apoptosis via flow cytometry. MDA-MB-231 were seeded in 12-well plates at a concentration of $2x10^5$ cells/well. Cells were allowed to attach overnight followed by synchronization in serum-free media. Cells were then treated with fresh media containing LOE at 0.15 and 0.30 mg/ml for 24 h in triplicate while controls were treated with media containing methanol. Cells collected via trypsinization and centrifuged and supernatant was removed. Next, 100 μl Muse™ Annexin V Dead Cell Assay stain (EMD Millipore) was added to the cell pellet and samples were resuspended by gentle pipetting. Samples were incubated in the dark for 20 min, following which they were run through a Muse™ Cell Analyzer (EMD Millipore) using manufacturer's protocol.

Caspase-3/7 activation assay. MDA-MB-231 cells were seeded at 10^5 cells/well in a 96-well plate and allowed to attach overnight. LOE and control treatments were prepared in fresh media containing 5 μM IncuCyte™ Caspase-3/7 apoptosis assay reagent and added to the cells, following which the plate was placed in an IncuCyte® ZOOM live-cell analyzer. Cells were imaged recurrently for 24 h and quantified for changes in caspase-3/7 activation via measurement of fluorescence intensity at a wavelength of 400 nm.

Western blotting. MDA-MB-231 cells were seeded at 10^6 cells/well in 6-well plates and allowed to attach overnight. Cells were then treated with fresh media containing LOE at 0.06 or 0.15 mg/ml for 3, 6, 12 and 24 h intervals. Treated cells were collected in RIPA buffer and sonicated to obtain lysates. Cell lysates were cleared of debris by centrifugation and protein concentration of lysates was estimated via the bicinchoninic acid (BCA) assay. Next, 20 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinyl difluoride (PVDF) membrane and immunoblotted. Primary antibodies for cyclin D1, cellular Inhibitor of Apoptosis Protein 2 (cIAP2), RIP1, cleaved caspase-8 and cleaved Poly-ADP Ribose Polymerase (PARP) were purchased from Cell Signaling Technologies, Danvers, MA, USA. Cleaved caspase-3 antibody was purchased from Epigenomics, WA. β-actin and β-tubulin antibodies were obtained from Sigma-Aldrich and Abcam, Cambridge, MA, USA, respectively. Quantification was carried out using ImageJ software (35).

Statistical analysis. Data are presented as mean values with corresponding standard errors from various experiments. Analysis for statistical significance for MTT assay was carried out using one-way analysis of variance (ANOVA). Analysis for statistical significance for western blotting, flow cytometry and caspase-3/-7 assay were carried out using Student's unpaired two-tailed t-test.

Results

LOE decreases viability of triple-negative breast cancer cells. In order to study the differential response of normal, invasive, and malignant triple-negative cells to LOE treatment, MCF10A, MCF10A-H, MDA-MB-231 and CRL-2321 cells were treated with increasing dosages of LOE for 24 h and changes in cellular metabolic activity were quantified using the MTT assay and utilized as a reflection of cell viability (Fig. 1).

Relative to control treatment, there was a dose-dependent decrease in cell viability of MDA-MB-231 cells. LOE at a concentration of 0.15 mg/ml resulted in 50% reduction in MDA-MB-231 cell viability while a higher dosage of 0.2 mg/ml resulted in a 95% reduction in MDA-MB-231 cell viability. Importantly, LOE impacted cell viability of MDA-MB-231, as well as the other triple-negative invasive and malignant cell lines (MCF10A-H and CRL-2321, respectively) to a much greater degree than in normal MCF10A mammary epithelial cells. In contrast with MDA-MB-231 cells, there was only a 20% decrease in cell viability of MCF10A cells with 0.15 mg/ml LOE treatment and a 40% decrease with 0.2 mg/ml LOE. Curiously, CRL-2321 cells demonstrated greater sensitivity than MDA-MB-231 cells in their initial response to low doses of LOE, but viability in this cell line did not decrease further with increasing dosage.

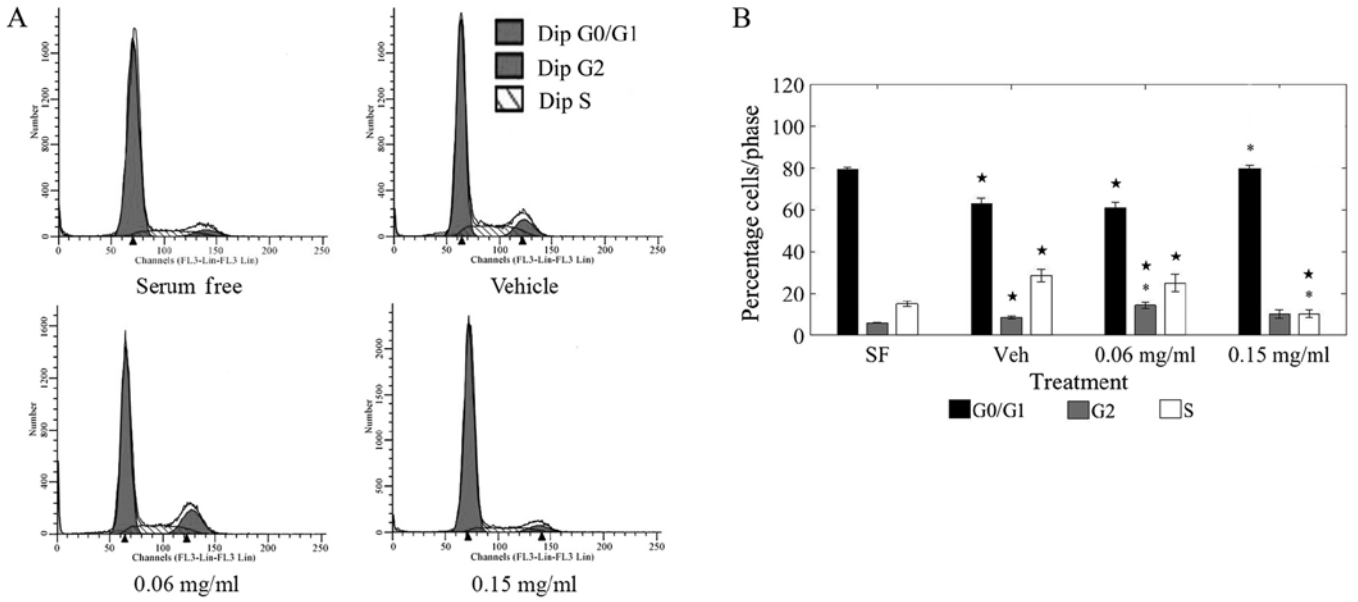

Figure 2. *Lippia origanoides* extract (LOE) induces G0/G1 phase arrest in MDA-MB-231 cells. MDA-MB-231 cells treated with indicated concentrations of LOE for 36 h were stained with propidium iodide (PI) and differential staining was measured using an FC500 flow cytometer (Bindley Flow Cytometry Facility). (A) Representative plots of raw data indicating cell cycle stages from treatment groups. (B) Average distribution across different cell cycle stages from various treatment groups. SF, serum-free treatment. n=3; *P<0.05, significantly different from vehicle treatment. *P<0.05, significantly different from serum-free treatment.

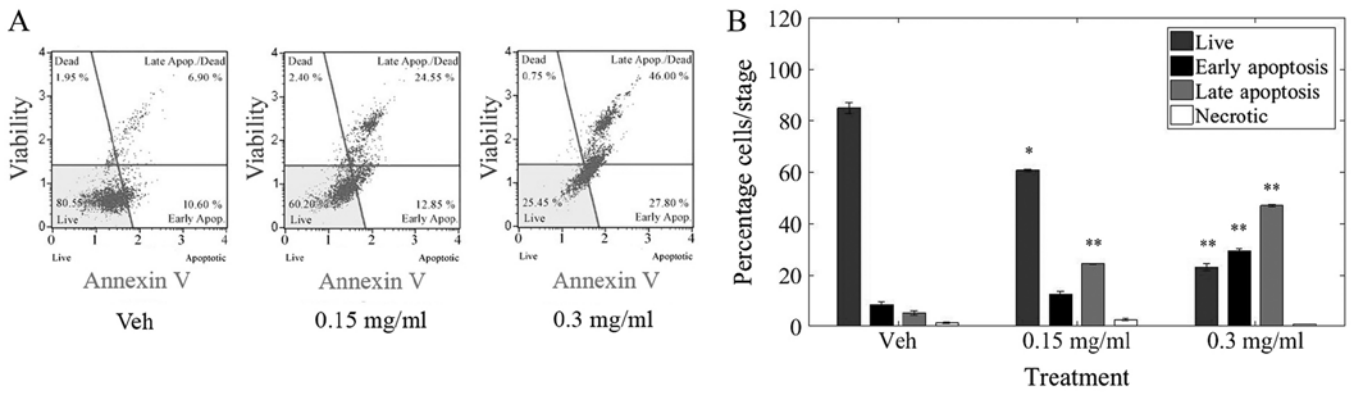

Figure 3. *Lippia origanoides* extract (LOE) induces apoptosis but not necrosis in MDA-MB-231 triple-negative breast cancer cells. MDA-MB-231 cells were treated with indicated concentrations of LOE for 24 h and stained with Annexin-V/7-Aminoactinomycin D (7-AAD). This was followed by measurement of differential Annexin-V/7-AAD staining using a Muse™ Cell Analyzer. (A) Representative plots of raw data showing distribution of cells as Live, Early Apoptotic, Late Apoptotic or Necrotic. (B) Quantified graph showing effect of LOE on apoptosis in MDA-MB-231 cells. n=3. Significant difference between LOE-treated cells and control (Vehicle-treated) cells is indicated as *P<0.05, **P<0.005.

LOE induces cell cycle arrest in triple-negative breast cancer cells. To quantify changes in cell cycle progression induced by LOE treatment, MDA-MB-231 cells were treated with LOE for 36 h. Control cells were treated with either serum-free media or growth media containing methanol. Cells were then collected and stained with propidium iodide followed by analysis using an FC500 flow cytometer. Representative raw data showing shifts in cell cycle phases under various treatments are shown in Fig. 2A.

Treatment with LOE at 0.15 mg/ml induced a significant shift away from the replication stage (S-phase) of the cell cycle as compared to vehicle-treated cells (Fig. 2B). Of the total cells counted, almost 20% shifted away from the S-phase and toward the G0/G1 phase upon LOE treatment at 0.15 mg/ml. Further, there was a striking resemblance in the S-phase distribution between 0.15 mg/ml LOE-treated cells and serum-starved cells (0.15 mg/ml LOE: 79.78% G0/G1; SF: 79.27% G0/G1), corroborating that LOE induces a halt in cell cycle progression in MDA-MB-231 cells.

LOE induces apoptosis in triple-negative breast cancer cells. Cell death may take the form of apoptosis, a programmed and controlled process involving the degradation and clearance of cellular constituents; or necrosis, a traumatic and inflammatory process which leads to the expulsion of cellular material into the extracellular environment (36). In order to analyze the impact of LOE on cell death in triple-negative breast cancer cells, MDA-MB-231 cells were treated with LOE for 24 h. Cells were stained with Annexin V/7-Aminoactinomycin D and evaluated in a Muse Cell Analyzer (Merck Millipore,

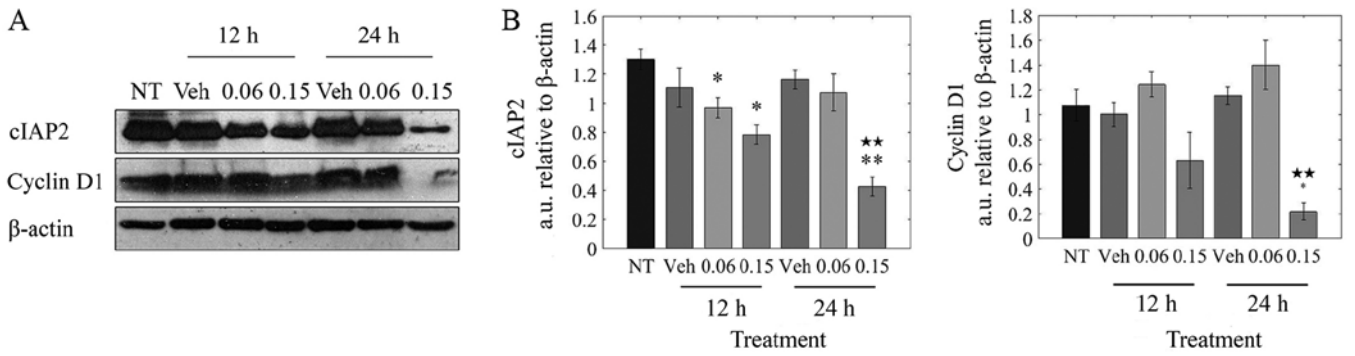

Figure 4. *Lippia origanoides* extract (LOE) inhibits markers of cell cycle progression and survival. MDAMB231 cells were treated with 0.06 and 0.15 mg/ml of LOE for various time intervals. Cells were then lysed in RIPA buffer. Lysates were subjected to western blotting and probed for indicated proteins. (A) Representative blots of cytostatic markers cyclin D1 and cIAP2. (B) Densitometry quantification of blots using ImageJ. Protein levels were normalized against β-actin and plotted as shown. n=3. Significant difference from untreated (NT) is indicated as *P<0.05 or **P<0.005; significant difference from Vehicle-treated (Veh) is indicated as *P<0.05 or **P<0.005.

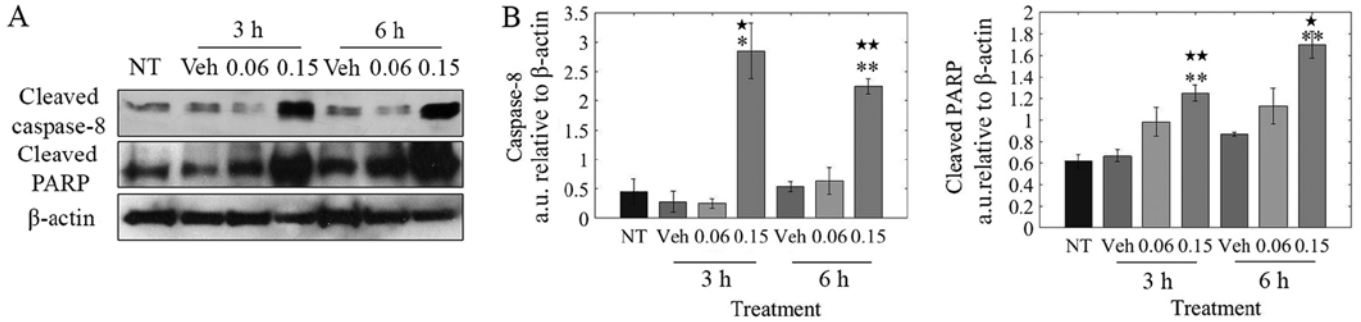

Figure 5. *Lippia origanoides* extract (LOE) induces apoptosis accompanied by caspase-8/-3 activation and PARP cleavage. MDA-MB-231 cells treated with LOE for various time intervals were lysed and subjected to western blotting and probed for indicated proteins. (A) Representative blots of apoptotic markers cleaved caspase-8, cleaved caspase-3 and cleaved PARP. (B) Densitometry quantification of blots using ImageJ. Protein levels were normalized against β-actin and plotted as shown. n=3. Significant difference from untreated (NT) is indicated as *P<0.05 or **P<0.005; significant difference from Vehicle-treated (Veh) is indicated as **P<0.005.

Billerica. MA, USA). Representative raw data from various treatments indicating shifts from live to early (EA) and late (LA) apoptosis as well as necrosis (Dead) is shown in Fig. 3.

Treatment with LOE induced a significant decrease in live cells as well a significant increase in apoptosis but not necrosis in MDA-MB-231 cells. Furthermore, 40% of cells were apoptotic after 24 h of 0.15 mg/ml LOE treatment while ~80% of cells underwent apoptosis after 0.3 mg/ml LOE treatment over the same time period.

LOE impacts critical regulators of cell cycle progression. As seen from Fig. 2B, LOE treatment induced a shift toward the G0/G1 phase and away from the S phase of the cell cycle in MDA-MB-231 cells. The progression from G0/G1 is mediated by cyclin D1, with cellular inhibitor of apoptosis (cIAP) proteins playing a supportive role by suppressing apoptosis (37,38). As observed in Fig. 4B, there was a significant (5-fold) decrease in cyclin D1 protein at 24 h post-treatment. cIAP2 levels were significantly reduced by 40% after 12 h and by ~65% after 24 h.

Taken together, these results suggest that the halt in cell cycle progression observed upon treatment of MDA-MB-231 cells with LOE is brought about by its impact on cyclin D1 and cIAP2 expression.

LOE induces apoptosis via caspase-8/-3 activation. Based on the observation that LOE induces apoptosis and not necrosis in MDA-MB-231 cells (Fig. 3B), our goal was to determine if LOE induces the extrinsic pathway of apoptosis via caspase-8 activation. As shown in Fig. 5B, LOE induced the rapid cleavage of caspase-8 within 3 h post-treatment with PARP cleavage peaking at 6 h post-treatment.

To further understand apoptotic signaling, MDA-MB-231 cells were analyzed for executioner caspase-3/-7 activity via live cell imaging using the IncuCyte ZOOM system. These studies showed a marked visible increase in caspase-3/-7 activation upon LOE treatment. As quantified in Fig. 6, there was a significant increase in caspase-3/-7 activity 3 h post-treatment with 0.15 and 0.3 mg/ml LOE, with peak activity at approximately 10 h post-treatment. This was supported by western blotting time-course data, which indicated a peak in caspase-3 activity at 12 h post-treatment with 0.15 mg/ml LOE (data not shown).

LOE reduces RIP1 protein levels. TNBC is often characterized by constitutive activation of NF-κB signaling via recruitment of key effector proteins by RIP1 (39,40). Immunoblotting revealed a significant decrease of ~40% in RIP1 protein levels in lysates from MDA-MB-231 cells treated with LOE for 9 h as compared to controls (Fig. 7B).

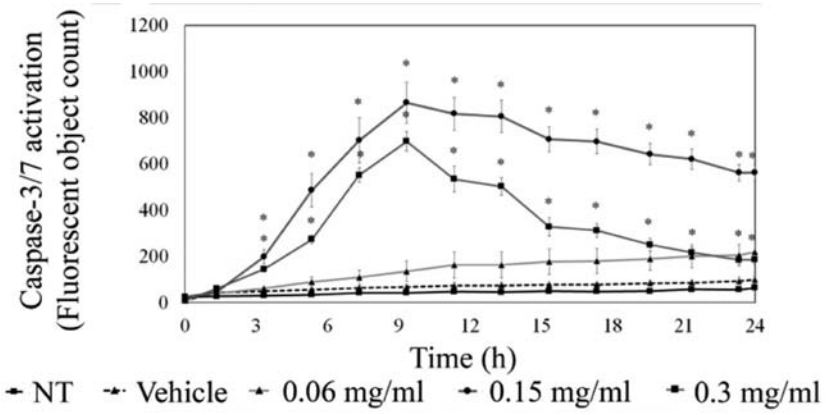

Figure 6. *Lippia origanoides* extract (LOE) induces executioner caspase-3/-7 activity in TNBC cells. MDA-MB-231 cells incubated with full growth medium containing indicated treatments and IncuCyte™ Caspase-3/7 apoptosis assay reagent were imaged periodically over 24 h in an IncuCyte® ZOOM live-cell analyzer to look at changes in caspase-3/-7 activity. Activity was quantified as the mean Green Fluorescent Object Count/Image for each treatment group and plotted as shown. n=5. *P<0.005, significantly different from control (Vehicle-treated) cells.

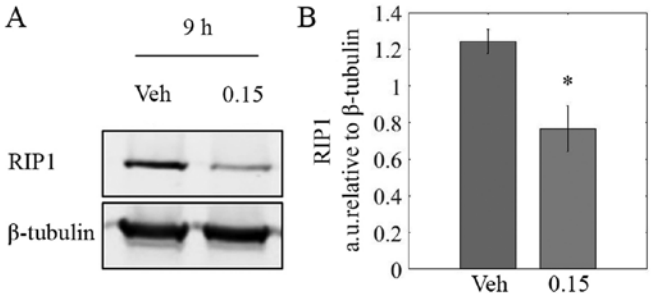

Figure 7. RIP1 protein levels are reduced upon treatment of TNBC cells with LOE. MDA-MB-231 cells were treated with 0.15 mg/ml LOE for 9 h and cell lysates were immunoblotted for RIP1. (A) Representative blots of RIP1, (B) densitometry quantification of blots using ImageJ. Protein levels were normalized against β-tubulin and plotted as shown. N = average of 3 replicates run twice. *P<0.05, significant difference from Vehicle-treated cells.

Discussion

TNBC continues to be a monumental concern in women's health issues. As our understanding of the mechanisms that govern the initiation and progression of breast cancer grows, it has become apparent that conventional chemotherapeutics are limited by their inability to target subtypes that are inherently more aggressive while being drug resistant. One such subtype is TNBC, which lacks expression of ER, PR and HER2/neu, thereby rendering TNBC resistant to conventional endocrine-targeted therapy. In this study, we provide support that an extract from the tropical South American plant *L. origanoides* (LOE) may be a possible source for therapeutic agents against TNBCs. In brief, we utilized the MTT metabolic activity assay to show LOE induces a dose-dependent decrease in the viability of MDA-MB-231 mammary adenocarcinoma cells, a universal standard *in vitro* model of highly invasive and drug resistant TNBC. This effect was much greater compared to normal-like MCF10A mammary epithelial cells (Fig. 1). Our observation that LOE also significantly reduced cell viability in triple-negative Hras-transformed MCF10A invasive cells as well as the CRL2321 cell-line, recently shown to be the best *in vitro* phenotypic representative of grade 3 TNBC tumors (41), further confirms its anti-TNBC effects. This observation suggests the LOE induces molecular actions that target pathways specific to cancer cells. We also note that while CRL-2321 cells have a greater sensitivity to low doses of LOE than MDA-MB-231 cells, higher dosages of LOE do not induce decreases in CRL-2321 viability beyond 80%. Previous studies have shown that cancer cell line subpopulations and sub-lines can exhibit a high degree of transcriptome and phenotypic variability leading to observable differences in metastatic activity (42) and drug sensitivity (43). This suggests the possibility of heterogeneity within subpopulations of the CRL-2321 cell line allowing for compensatory survival pathways providing modest resistance to LOE treatment. The identification of novel mechanisms targeted by new small molecular inhibitors is of major interest in our continued studies of the actions of LOE.

Our study also quantified the molecular influences of the extract within cancer cells. Cyclin D1 is a protein which associates with cyclin-dependent kinase-4 and -6 (CDK-4 and -6) to form heterodimers that progress the cell cycle from G0/G1 to S phase (37). In addition, cellular inhibitor of apoptosis proteins (cIAPs) prevent apoptosis and permit cell cycle progression via binding and possibly inactivating executioner caspases, i.e., caspase-3 and -7 (38). cIAP2, is known to be constitutively expressed in MDA-MB-231 cells, and has been shown to protect against death-inducing ligands such as TNF-α by possibly enhancing NF-κB signaling through RIP1 activation as well as by binding and inhibiting caspase-3 (39). Herein, we observed that LOE induced G0/G1 phase halt in MDA-MB-231 cells (Fig. 2). Western blot analysis revealed the effects of LOE on cell cycle progression could be explained by a reduction in cyclin D1 and cIAP2 levels upon treatment of the cells with the extract (Fig. 4). These experiments begin to build an important framework for the actions of LOE on cell cycle regulation.

We next studied LOE's impact on MDA-MB-231 cell death. Upon triggering of the extrinsic pathway of apoptosis, procaspase-8 is activated via dimerization-induced auto-cleavage and subsequently cleaves and activates the master 'executioner' caspase, caspase-3 (44,45). We revealed a potential mechanism behind the actions of the extract by showing LOE-induced apoptosis involved activation of caspase-8/-3 and cleavage

of PARP (Figs. 5 and 6), hallmarks of extrinsic apoptotic pathway activation (36). Additionally, LOE induced apoptosis in these cells unaccompanied by necrotic cell death (Fig. 3). As cancer therapy is focused on preventing damage to non-targeted tissue areas, this result provides additional support for LOE as a source for potential therapeutics with desirable characteristics.

In previous work, Stashenko *et al* have characterized the major components of LOE (28). However, it remains to be defined which component, or combination of components, is responsible for the extract's apoptotic effects. Furthermore, it is imperative that major cellular pathways involved in LOE signal transduction be delineated. Based on previous studies involving components present in the LOE, one possible candidate pathway central to LOE-induced apoptosis is NF-κB signaling. Constitutive activation of this pathway has been previously linked to triple-negative breast cancer and blocking NF-κB signaling can spontaneously induce cell death via a caspase-8/-3-dependent mechanism or render cells susceptible to pro-apoptotic signals (40,46,47). In normal cells, ligand binding to TNFR1 is followed by rapid activation of the extrinsic pathway of apoptosis. However, in contrast to normal cells, ligand binding to TNFR1 in TNBC cells instead activates NF-κB signaling via membrane-recruitment of the scaffold protein RIP1, a Ser/Thr kinase (39). In brief, cIAP2 exerts E3 ubiquitin ligase activity to polyubiquitinate and activate RIP1, which goes on to phosphorylate the IKKα/β-NEMO complex. Active IKKs then phosphorylate IκB proteins which sequester p50/RelA (NF-κB) dimers to the cytoplasm, targeting the IκBs for polyubiquitination and subsequent degradation. This frees p50/RelA to translocate to the nucleus and act as a transcription factor for pro-survival genes such as cyclin D1 and cIAPs (48,49).

We hypothesized that components of LOE could interfere with NF-κB signaling, leading to a halt in the cell cycle and induction of apoptosis. Indeed, we showed LOE treatment resulted in a 40% decrease in protein levels of RIP1 in MDA-MB-231 cells (Fig. 7B). This may, at least in part, be explained by our previous observation that LOE treatment induced activation of caspase-8. Active caspase-8 has previously been shown to cleave RIP-1, thereby inhibiting pro-survival NF-κB signaling and inducing apoptosis (50). This result, coupled with our findings that LOE inhibits cyclin D1 and cIAP2, proteins activated by NF-κB signaling, while simultaneously stimulating caspase-8 dependent cell death, a characteristic of NF-κB inhibition, sheds critical light on the mechanistic foreground of LOE action. All together, these data highlight the potential of *L. origanoides* extract and its major constituents as cancer therapeutics.

In summary, we have shown that LOE promotes apoptosis in TNBC cells, while having a much reduced impact on cell death in normal mammary cells. We also found that LOE reduces cell viability, alters the cell cycle and primarily leads to tumor cell apoptosis and not necrosis. Further, we established some of the mechanisms of LOE actions, showing that it suppresses markers of cell cycle progression and cell survival while inducing the extrinsic pathway of apoptosis via caspase-8/-3 activation. Finally, we revealed that levels of RIP1, an upstream effector of pro-survival NF-κB signaling, are reduced upon LOE treatment. These data collectively support that LOE can be a valuable source for development of novel anticancer agents. Moving forward, beyond fully describing the cellular mechanisms of LOE action, a top priority will be identifying the major component or combination of components responsible for its bioactivity. Plant-derived compounds offer a vast source of small molecule inhibitors that could be used both in their native state as well as chemically modified to optimize their pharmacological activity. The bio-reactivity of the LOE-treatment in TNBC cells *in vitro* supports the idea that future treatments for individual cancer subtypes lie in naturally-derived chemicals.

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