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Anti-proliferative effect of *Annona* extracts on breast cancer cells

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Abstract: Backgorund: Fruits and seed extracts of *Annona montana* have significant cytotoxic potential in several cancer cells. This study evaluates the effect of *A. montana* leaves hexane extract on several signaling cascades and gene expression in metastatic breast cancer cells upon insulin-like growth factor-1 (IGF-1) stimulation. **Methods:** MTT assay was performed to determine the proliferation of cancer cells. Propidium iodide staining and flow cytometry analysis of Annexin V binding was utilized to measure the progression of the cell cycle and the induction of apoptosis. Protein expression and phosphorylation were determined by western blotting analysis to examine the underlying cellular mechanism triggered upon treatment with *A. montana* leaves hexane extract. **Results:** *A. montana* leaves hexane (sub-fraction V) blocked the constitutive stimulation of the PI3K/mTOR signaling pathways. This inhibitory effect was associated with apoptosis induction as evidenced by the positivity with Annexin V and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNNEL) staining, activation of caspase-3, and cleavage of PPAR. It also limited the expression of various downstream genes that regulate proliferation, survival, metastasis, and angiogenesis (i.e., cyclin D1, survivin, COX-2, and VEGF). It increased the expression of p53 and p21. Interestingly, we also observed that this extract blocked the activation of AKT and ERK without affecting the phosphorylation of the IGF-1 receptor and activation of Ras upon IGF-1 stimulation. **Conclusion:** Our study indicates that *A. montana* leaves (sub-fraction V) extract exhibits a selective anti-proliferative and proapoptotic effect on the metastatic MDA-MB-231 breast cancer cells through the involvement of PI3K/AKT/mTOR/S6K1 pathways.

Introduction

Based on the World Health Organization (WHO) report, more than 80% of the world population depends on traditional and alternative medicine for their primary healthcare needs (Adams, 2007; Sharifi-Rad *et al.*, 2019; WHO, 2019). More than 60% of used anti-cancer drugs originate from a natural source. Still, their active constituents and cellular/molecular targets have yet to be fully understood. The anti-cancer activity of most natural products is often related to regulating immune function, inducing apoptosis or autophagy, or inhibiting uncontrolled cell growth and invasion (Huang *et al.*, 2021; Yang *et al.*, 2021; Lederer and Huber, 2022). The anti-cancer activity of most natural products is often related to regulating signaling transduction pathways, inducing apoptosis, or inhibiting uncontrolled cell growth and invasion (Sharifi-Rad *et al.*, 2019; Huang *et al.*, 2021). Cell signal transduction is regulated by various cell surface receptors (i.e., insulin growth factor-1 receptor (IGF-1R)). IGF-1 plays a crucial role in cellular physiology by modulating several signaling pathways, such as the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR). These are essential enzymes regulated via several intracellular stimuli and control key cellular functions, such as proliferation, survival, and growth (Sharma *et al.*, 2022; Zhang *et al.*, 2022). Several species of genus *Annona*, in particular, known for a range of biomedical applications as well as in folk medicine, have been investigated (Rupprecht *et al.*, 1990; Chang and Wu, 2001; Pardhasaradhi *et al.*, 2005; Lim, 2012; Pratheeshkumar

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et al., 2012; Ribeiro et al., 2013; Formagio et al., 2015; Kumar et al., 2015; Tundis et al., 2017). Several studies have been conducted on *A. muricata* and *A. squamosa*, focusing on their anti-cancer properties against different cancer cell lines (Wang et al., 2014; Syed Najmuddin et al., 2016). Other studies revealed the anti-proliferative effect of *A. cherimola* leaves and seeds on cancer and leukemic cells (Suresh et al., 2011; Arunjyothi et al., 2012; Haykal et al., 2019). Interestingly, aqueous and methanol extracts from *A. muricata* leaves lowered the blood glucose level and showed antioxidant effects on diabetic rats (Adeyemi et al., 2008). Recent studies revealed the anti-proliferative effect of *A. montana* fruit on several cancer cell lines. Extracts from its fruits, seeds, and leaves have significant cytotoxic potential in several cancer cells (Wu et al., 1995; Wang et al., 2001; Chuang et al., 2008; Alvarez Colom et al., 2009; Bailon-Moscoso et al., 2016). Furthermore, methanol, hexane, and ethyl acetate extracts from *A. montana* reduced the formation of fat cells (Leung et al., 2022). Administering extracts prepared from *A. squamosa* also inhibits glycemic and lipemic indices and the levels of the antioxidant enzymes (i.e., glutathione reductase) (Gupta et al., 2005; Kaleem et al., 2006). However, there are currently no studies on the effect of extracts from the *A. montana* leaves on the signaling transduction pathways driven by IGF-1 in breast cancer cells. Thus, due to the vital role of IGF-1 on PI3K-Akt-mTOR and MAPKs signaling pathways in cellular functioning and pathologic modification (AsghariHanjani and Vafa, 2019), we decided to investigate whether hexane extracts of *A. montana* utilize their anti-cancer effects by modulating the PI3K/AKT/mTOR/S6K1 and MAPK signaling driven by IGF-1 in breast cancer cells. Thus, natural compounds in *A. montana* extracts could lead to the dysregulation of gene expression and inhibition of cell proliferation that affect apoptosis and promote proliferation, migration, and invasion in cancer cells. In addition, we analyzed the anti-proliferative effects of *A. montana* extracts on several human tumor cell lines.

Materials and Methods

Chemicals, reagents, and antibodies

All cell lines (HeLa, HepG2, A549, A431, MCF-7, PCS-600-010, MDA-MD-231, MCF-7, and SK-MEL-28) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagles medium-high glucose (DMEM), penicillin/streptomycin, and L-glutamine were purchased from Mediatech, Inc. (Manassas, VA). Unless otherwise indicated, all antibodies were acquired from Cell Signaling Technology (Boston, MA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Sigma-Aldrich (St. Louis, MO). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). IGF-1 was purchased from Cell Signaling Technology (Beverly, MA). Inhibitors (i.e., Wortmannin, PF-3758309, FR-180204) were obtained from Cayman Chemical. ISIS2503 oligonucleotide inhibitor was prepared as previously described (Chen et al., 1996). Other chemicals were obtained from Sigma unless otherwise stated.

Preparation of Annona montana *extract*

A. montana leaves were collected from the Redland region (Possum Trot Tropical Farm) in Miami, Florida, USA, by Dr. M.A. Barbieri and Mr. R. Barnum. Our botanist verified the taxonomic identity at the herbarium of the Department of Biological Sciences at Florida International University (Voucher: FTG182701, Fairchild Herbarium). The leaves of *A. montana* were separated from one individual plant and cleaned using running tap water. Then, the leaves were oven-dried in a convection oven at 60°C for 32 h. Dried leaves (2000 mg) were cut into small pieces and extracted with methanol (100 mL) in a 250 mL round bottom flask connected to a reflux condenser heated at 65°C for 30 min. The plant materials were filtered and washed twice with methanol (25 mL). All materials (extract and washes) were collected and pooled. Evaporation of the methanol extract by rotavapor/desiccation yielded a residue of 1180 mg for *A. montana* methanol extract (AmMe). Then each methanol extract was either dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 100 mg/mL plant extract or partitioned with hexane, ethyl acetate, and water, respectively, as described early (Chin et al., 2014). An aliquot (518 mg *A. montana* methanol extract) of the residue from the methanol extraction was partitioned between methanol and hexane. The hexane was removed from the methanolic phase and treated as previously described (Priestap et al., 2014). Finally, the residue was partitioned between water and ethyl acetate. All fractions collected (i.e., hexane-AmH-, ethyl acetate-Amea- and water-Amw-) were then individually evaporated and concentrated by rotorvapor/desiccation as previously described (Priestap et al., 2014). The hexane, ethyl acetate, and water extracts yielded 223, 102, and 95 mg of residue for *A. montana*, representing 82% of the initial methanol extract. A pool of several hexane fractions was collected (1000 mg), and was further fractionated using gravity column chromatography with hexane-ethyl acetate to increase polarity up to 90% ethyl acetate, then 100% ethyl acetate yielded twelve sub-fractions of increasing polarity (F1-F12). Each sub-fraction was dried, dissolved in DMSO, and tested for activity cell proliferation, cell cycle, invasion, colony formation, and Western blotting analysis.

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analyses

GC/MS determinations were carried out in a Hewlett Packard model 6890 instrument coupled to a Q-Mass 910 quadrupole selective detector (Perkin-Elmer) as previously described (Chin et al., 2014; Priestap et al., 2014). GC/FID determinations were performed on a Trace GC Ultra apparatus (Thermo Electron Corporation), and the output was recorded using a ChromQuest version 4.1 data system. Analyses were performed on capillary columns DB-5MS at the abovementioned conditions (Adams, 2007; Araujo et al., 2007; Babushok et al., 2011; Yan et al., 2013).

Cell culture

All cell lines were cultured according to standard mammalian tissue culture protocols and sterile technique as indicated by ATCC. Cell lines were cultured for less than 2 months, splitting them twice a week in a humidified incubator with 5% CO_2 at 37°C. Hela, SK-MEL-28, A431, A549, HepG2, MDA-MB-231, and MFC-7 cells were grown in DMEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin. MCF-10A cells were grown in DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 10 ng/mL human epidermal growth factor (hEGF), 100 ng/mL cholera toxins, 5 µg/mL bovine insulin and 500 ng/mL hydrocortisone. PCS-600-010 cells were grown in mammary epithelial cell basal medium supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 100 ng/mL recombinant human transforming growth factor-alpha (rhTGF-α), 5 µg/mL Apotransferrin, 1 µM epinephrine, 5 µg/mL recombinant human insulin (rhI) and 100 ng/mL hydrocortisone. Cells were harvested at approximately 80% confluence and then plated for subsequent passage and treatments.

Cell cycle analysis

Cell cycle progression was determined by flow cytometry analysis (Alvarez *et al.*, 2010). Cells were incubated in the absence or presence of *A. montana* extract for 24 h. A suspension of cells was fixed with methanol (70%) at 4°C, washed, and stained with propidium iodide (PI). Cells were analyzed with an Acurri-C6 Flow Cytometer (BD Biosciences, San Diego, CA), and cell cycle progression was measured with the Modfit LT program (Verity Software House, Topsham, ME).

Apoptosis assay

The Annexin V/fluorescein isothiocyanate (FITC) assay was performed using Annexin V Kit (BD Pharmigen, USA) to analyze the potential of *A. montana* extract in causing apoptosis. (The Annexin V signal indicates cellular apoptosis, while PI staining indicates necrotic or late apoptosis) (Elmore *et al.*, 2016). The cells were seeded in a 6-well plate at a concentration of 2.4×10^5 cells/mL and incubated overnight. The next day, the seeded cells were treated with *A. montana* extract and incubated for 24, 48, and 72 h. The cells were harvested at a time point, and the resulting pellets were resuspended in a binding buffer. To stain the cell suspension, 25 µL of FITC Annexin V and 5 µL of PI were added and allowed to stand in a dark place at room temperature for 15 min. Afterward, the stained cells were analyzed by an Acurri-C6 Flow Cytometer (BD Biosciences, San Diego, CA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Cells were seeded onto 6-well plates at a density of 1×10^6 cells per well, incubated for one day, and then treated with *A. montana* extract for 24 h. The cells were collected and washed with 1 mL phosphate buffer saline (PBS). Cells were fixed with paraformaldehyde for 30 min at room temperature, washed, and then permeabilized by 0.2% Triton X-100 for 15 min. After incubation, cells were washed and resuspended in 1 mL of PBS containing TUNEL solution (de Felice *et al.*, 2019) and incubated for 1 h at 37°C in the dark. Afterward, the stained cells were analyzed by an Acurri-C6 Flow Cytometer (BD Biosciences, San Diego, CA).

Wound healing assay

Cells (3.5×10^5) were seeded in a 6-well plate and incubated overnight. To evaluate migration (non-directional migration) the next day, a straight wound line was drawn across the 100% confluent attached cell layer with pipette tips as previously described (Cappiello *et al.*, 2018). Plates were washed to remove unattached cells with PBS and replaced with a fresh DMEM medium. *A. montana* extract was added to the wells, and images of the closure of the wound were recorded at 24 h using the inverted microscope equipped with a camera.

Cell proliferation assay

Cells were seeded in 96-well plates at 2×10^3 cells/well density in 100 µL medium and incubated overnight. The next day, the seeded cells were treated with *A. montana* extract or subfraction and incubated for 8 h (for extract) or 24, 48, and 72 h (for subfraction). After the incubation, the MTT solution (5 mg/mL) was added to the plates, and the cells were incubated at 37°C for 6 h. The formazan, derived from MTT by living cells, was dissolved in 10% SDS (150 µL per well), and the absorbance was measured at OD_{595} nm as described (Mosmann, 1983). All MTT experiments were performed in duplicate and repeated at least three times.

Cell migration and invasion assays

Cells (~70% confluent) were serum-starved for 24 h. Then, cells were seeded (2×10^5 cells/mL) in the insert chamber coated with solidified Matrigel (BD Biosciences) for the invasion assay, whereas, for the migration assay (directional migration), the Matrigel basement membrane did not coat the chamber. In the lower compartment of the chamber, 2 mL of DMEM supplemented with 10% FBS and the desired concentration of *A. montana* extract were added. The inserts were incubated in a 37°C CO_2 incubator for 24 h. The inserts were removed afterward, and the inner side of the inserts was swabbed to remove the non-invaded cells. The outer side of the inserts bearing the migrated/invaded cells was then fixed in methanol for 30 min before being stained with 0.01% of crystal violet. Images of the membranes were acquired or counted under an inverted microscope with a camera (×100). The dye was extracted, and the absorbance reading was obtained using a spectrophotometer (Hulkower and Herber, 2011).

Anchorage-independent colony formation assay

For measurement of anchorage-independent growth, cells were trypsinized and resuspended in a culture medium containing 0.9% methylcellulose (Fluka, Saint Louis, MO) in DMEM containing 5% FBS. Aliquots of 1 mL containing 2×10^3 cells were plated on 35-mm bacteriological dishes in the absence or presence of *A. montana* extract containing 5% FBS. As previously described, colonies were counted under an inverted microscope after 14–16 days (Borowicz *et al.*, 2014).

Cell viability assay

Cells were grown in a 48-well culture plate in DMEM with 10% FBS containing antibiotics and then incubated with either DMSO vehicle or *A. montana* extract for 24 h. After incubation, the released lactate dehydrogenase (LDH) into the culture supernatant was measured by the colorimetric LDH Cytotoxicity Assay (BioVision) according to the manufacturer's instructions (Smith *et al.*, 2011).

Western blotting assay

To prepare cell lysates, cell monolayers were washed with ice-cold lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na2 EDTA, 1% NP40, and 0.1% Na deoxycholate) containing both protease and phosphatase inhibitors. Lysates were collected by centrifugation, and protein concentrations were estimated by using the BCA protein assay (Thermo Fisher Scientific, Inc., Pittsburgh, PA) following the manufacturer's instructions. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes, which were blocked, probed with the specific antibodies, and then visualized by western blotting analysis. Relative levels of MMP-2 and MMP-9 proteins were determined by densitometry using the ratio of MMP-2 and MMP-9 to GAPDH. Relative levels of PI3K, AKT, SGK1, JNK, ERK, mTOR, p38, and RAF-1 were determined using the ratio of p-PI3K, p-AKT, p-SGK1, p-JNK, p-ERK, p-mTOR, p-p38, p-RAF-1 to t-PI3K, t-AKT, t-SGK1, t-JNK, t-ERK, t-mTOR, t-p38, and t-RAF-1, respectively. Relative levels of survivin, IAP-1, IAP-2, Cyclin-D1, COX-2, VEGF, Caspase, PARP, p53, IGF-1R, and p21 proteins were determined by densitometry using the ratio of these proteins to GAPDH as previously described (Tall *et al.*, 2001).

Ras activity analysis

Cells were lysed using a buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 5% (v/v) glycerol, and 1% (v/v) Triton-X-100 supplemented with 2 mM phenylmethylsulfonyl fluoride (lysis buffer). Lysates (1 mL) were then incubated with 100 µL of glutathione beads containing 10 µg of GST-RAF-RBD at 4°C while rocking for 1 h. After incubation, the beads were washed three times using Hank's balanced salt solution (HBSS) with 5 mg/mL bovine serum albumin (BSA) (HBSS-BSA). The pull-downs were subjected to SDS-PAGE and analyzed by western blotting using an anti-Ras antibody as previously described (Camalier *et al.*, 2010).

Statistical analysis

All experiments were done in duplicates, and they were repeated at least three times. Values are represented as the standard error of the mean of triplicates (SEM), and the statistical significance was analyzed by two-way ANOVA or two-tail student's *t*-test. Results with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were statistically significant.

Results

Anti-proliferative effect of Annona montana *extracts on several types of cancer cell lines*

Cancer cells must continuously refurbish cellular metabolism to fulfill the demands of growth and proliferation (Fares *et al.*, 2020). Cell proliferation was determined by comparing the

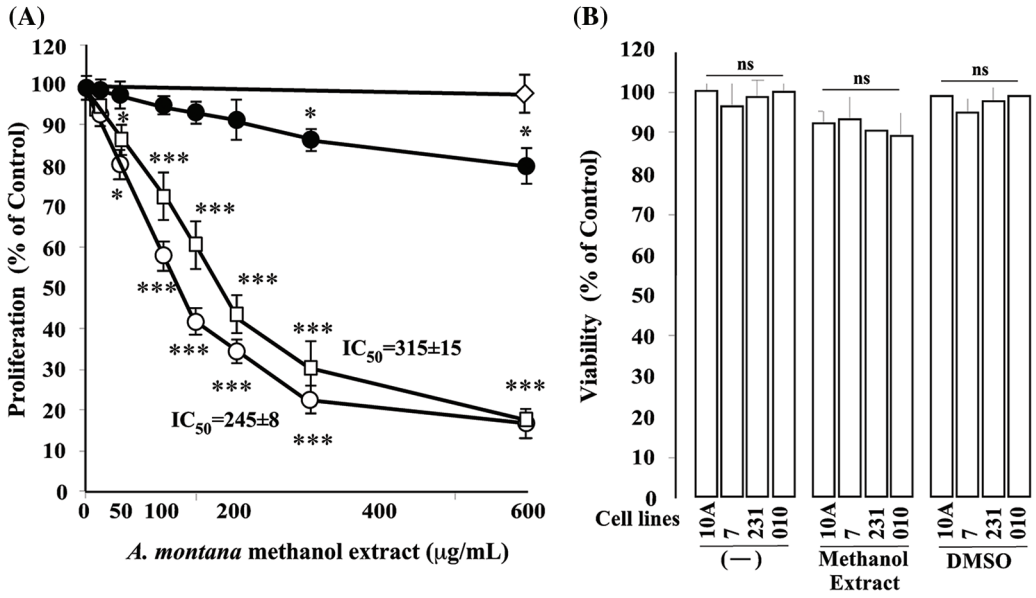

FIGURE 1. A dose-dependent inhibitory effect of *Annona montana* methanol extract on cell proliferation. (A) Cells (MCF-10A [●], MCF-7 [○], and MDA-MB-231 [□]) were treated with several concentrations (0 to 600 µg/mL) of *A. montana* methanol extract for 8 h, respectively. Then, cellular proliferation was quantified spectroscopically using the MTT assay described in the Material and Methods. Dimethyl sulfoxide (0.01%) [◊] was used as a control with MCF-7 cells. IC50 was expressed as µg/mL. Data represent the mean ± SEM of three independent experiments. Results were expressed as a percentage of non-treated cells (control cells). $^{*}p < 0.05$ and $^{***}p < 0.001$ as determined by two-way ANOVA followed by Tukey's *post hoc* test. (B) Cells [MDA-MB-231(231), MCF-10A (10A), MCF-7 (7) PCS-600-100 (010)] were cultured in medium alone and containing either 0.01% dimethyl sulfoxide or 600 µg/mL of *A. montana* methanol extract for 8 h. Cell viability was determined by the LDH assay. All values are presented as the mean ± SEM of three experiments performed in triplicate. ns: it means not significant.

survival of cells for 24 h in the untreated cells, which was normalized to 100%. Cells were treated once with different concentrations of three types of *A. montana* extracts (i.e., *A. montana* methanol, hexane, ethyl acetate, and water). Fig. 1A shows that the *A. montana* methanol extract inhibited the proliferation of human tumorigenic MCF-7 and metastatic MDA-MB-231 breast cancer cell lines. Specifically, the *A. montana* methanol extract shows an IC_{50} of 315 ± 15 μg/mL for MDA-MB-231 cells and an IC_{50} of 245 ± 8 μg/mL for MCF-7 cells, respectively. Fig. 1A also shows the effect of the *A. montana* methanol extract on the proliferation of MCF-10A, which was considerably lower. It only inhibited 19.5 ± 2.2% at 600 μg/mL. Consistent with this observation, we found that this *A. montana* methanol extract was unable to affect the viability of PCS-600-010 primary normal mammary epithelial cells (compare the viability of non-treated cells (92 ± 8%) with cells treated with 600 μg/mL of *A. montana* methanol extract (90 ± 6% cell viability) as evidenced by the quantification of LDH as described in Material and Methods section. Furthermore, adding 0.01% DMSO did not affect the cell viability of any of the treated cell lines (Figs. 1A and 1B). As a positive

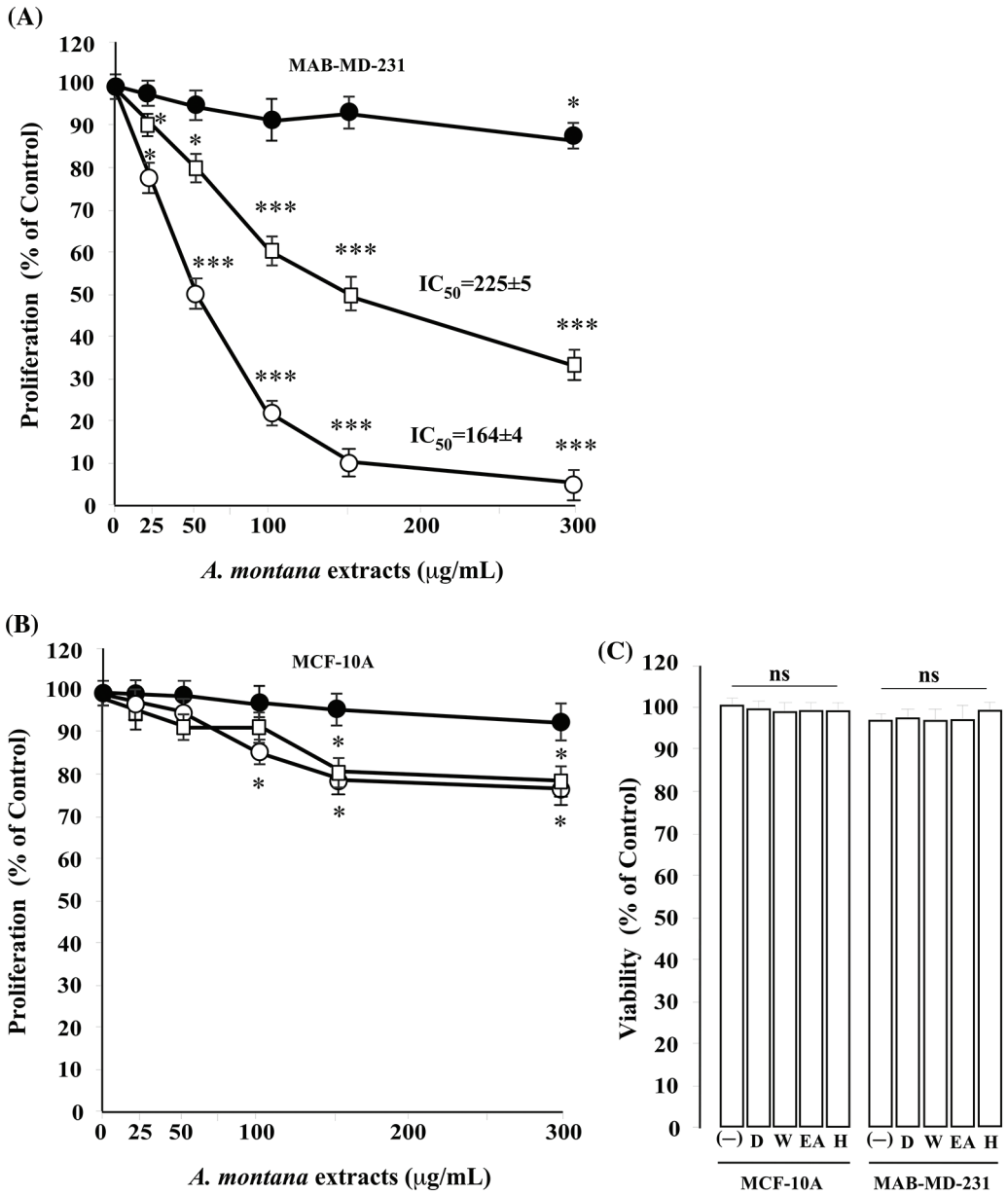

FIGURE 2. Effect of hexane, ethyl acetate, and water *Anonna montana* extracts on cell proliferation. Cells ((A) MDA-MB-231 (B): MCF-10) were treated with several concentrations (0 to 300 μg/mL) of hexane (○), ethyl acetate (□), and water (●) fractions of *A. montana* methanol extract for 8 h. Then, cellular proliferation was quantified spectroscopically using the MTT assay described in the Material and Methods section. IC50 was expressed as μg/mL. Data represent the mean ± standard error of three independent experiments' mean (SEM). Results were expressed as a percentage of non-treated cells (control cells). $^{*}p < 0.05$ and $^{***}p < 0.001$ as determined by two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. (C) Cells (MDA-MB-231 and MCF-10A) were cultured in medium alone (-) and containing either 0.01% dimethyl sulfoxide (D) or 300 μg/mL of *A. montana* extracts (e.g., hexane-H, ethyl acetate-EA, water-W) for 8 h. Cell viability was determined by the LDH assay. All values are presented as the mean ± SEM of three experiments performed in triplicate. $^{*}p < 0.05$ and $^{***}p < 0.001$ as determined by two-way ANOVA followed by Tukey's *post hoc* test.

TABLE 1

Effect of Hexane sub-fractions (40 µg/mL) on the proliferation of MDA-MB-231 after 24 h of incubation generated from MTT assay

Hexane subfractions	Proliferation (% of control ± SEM)
I	91 ± 4
II	88 ± 4
III	93 ± 5
IV	95 ± 4
V	31 ± 2***
VI	88 ± 6
VII	69 ± 4*
VIII	91 ± 5
IX	93 ± 3
X	100 ± 2
XI	100 ± 2
XII	93 ± 5
Control	100 ± 2

Note: Each data was expressed as mean ± standard error of triplicate determinations' the mean (SEM). *Statistical significance ($p < 0.05$) between the control and sub-fraction hexane VII-treated group in 24-h. ***Statistical significance ($p < 0.001$) between the control and sub-fraction hexane V-treated group in 24 h.

control, we used wortmannin, a potent inhibitor of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activity, which also inhibited the proliferation of MDA-MB-231 and MCF-7 cells with 43 ± 9% and 57 ± 5% of inhibition, respectively. Furthermore, the addition of wortmannin (100 µg/mL; 0.233 µM) did not significantly affect the viability of MCF-10A cells (96 ± 4% cell viability) as compared with control cells (100 ± 3% cell viability), and it is consistent with our previous observations (Chin *et al.*, 2014). These results suggest a strong activity in inhibiting the proliferation of the *A. montana* methanol extract on these cancer cells. To obtain additional information about the nature of the active compound in *A. montana* leaves, the methanol extract was subsequently fractionated as described in the Materials and Methods section. Three fractions (named hexane, ethyl acetate, and water fractions) were obtained. The *A. montana* hexane fraction exhibited the most potent inhibitory effect (IC_{50} 164 ± 4 µg/mL) on cell proliferation of MDA-MB-231 cells as compared with the *A. montana* ethyl acetate fraction, which showed inhibition of cell proliferation with IC_{50} 225 ± 5 µg/mL (Fig. 2A). Interestingly, the aqueous fraction did not show any effect (Fig. 2A), and more importantly, these *A. montana* fractions showed a limited inhibitory effect on the proliferation of MCF-10A cells (Fig. 2B). As expected, these fractions did not show a significant effect on the viability of MDA-MB-231 cells (compare the viability of non-treated cells (94 ± 6% cell viability) and 0.01% DMSO-treated cells (93 ± 5% cell viability) with cells treated with 300 µg/mL of *A. montana* hexane (94 ± 5% cell viability), ethyl acetate (95 ±3% cell viability) and water extracts (98 ± 2% cell viability) as evidenced by the quantification of LDH as described in Material and Methods (Fig. 2C). We also fractionated the hexane fraction with a combination of Hexane: Ethyl acetate in order of increasing polarity. Each sub-fraction was tested for its activity on cell proliferation. As shown in Table 1, we observed that *A. montana* hexane subfraction V, and in less extent, fraction VII, strongly decreased the proliferation of MBA-MD-231 cells. On the other hand, the IC_{50} results for the anti-proliferative effect of the *A. montana* hexane sub-fraction V on several types of cancer cell lines (i.e., HeLa, PC-3, HepG2, A549, A431, SK-MEL-28, MDA-MB-231, and MCF-7) are shown in Table 2. The proliferation of these cancer cell lines was susceptible to *A. montana* hexane subfraction V. A549, and SK-MEL-28 cell lines were the least affected by adding *A. montana* hexane subfraction V (i.e., high IC_{50}). In contrast, the proliferation of HeLa and MCF-7 cell lines showed high activity inhibiting cell proliferation as they have lower IC50 values. Interestingly, HeLa and MCF-7 cell lines were the most affected after 72 h of treatment with *A. montana* hexane subfraction V. We also considered the possibility that the extent of inhibition is time-dependent. Thus, we then determine the effect of adding *A. montana* hexane subfraction V on the

TABLE 2

Effect of hexane sub-fraction V on the proliferation of several cell lines

Cell line	Duration (h)	IC_{50} (µg/mL)
HeLa	24	20.2 ± 1.5
	48	14.2 ± 1.2*
	72	6.8 ± 0.8***
HepG2	24	37.2 ± 1.5
	48	34.2 ± 1.4*
	72	12.8 ± 1.1***
A549	24	50.7 ± 2.1
	48	41.7 ± 1.5*
	72	21.2 ± 1.1***
A431	24	28.2 ± 1.4
	48	25.7 ± 2.1 *
	72	11.8 ± 1.5***
SK-MEL-28	24	40.5 ± 2.4
	48	34.7 ± 2.1*
	72	27.8 ± 2.5***
MDA-MB-231	24	31.5 ± 1.5
	48	22.7 ± 1.7 *
	72	8.8 ± 1.2***
MCF-7	24	20.5 ± 0.8
	48	12.2 ± 1.7*
	72	5.8 ± 1.5***

Note: IC_{50} of the hexane sub-fraction V on the proliferation of several cancer cells after 24, 48, and 72 h of incubation was generated from the MTT assay. Each data was expressed as mean ± standard error of triplicate determinations' the mean (SEM). *Statistical significance ($p < 0.05$) between control and hexane-treated group in 48-h time-point by student's *t*-test. ***Statistical significance ($p < 0.001$) between the control and hexane-treated group in 72-h by student's *t*-test.

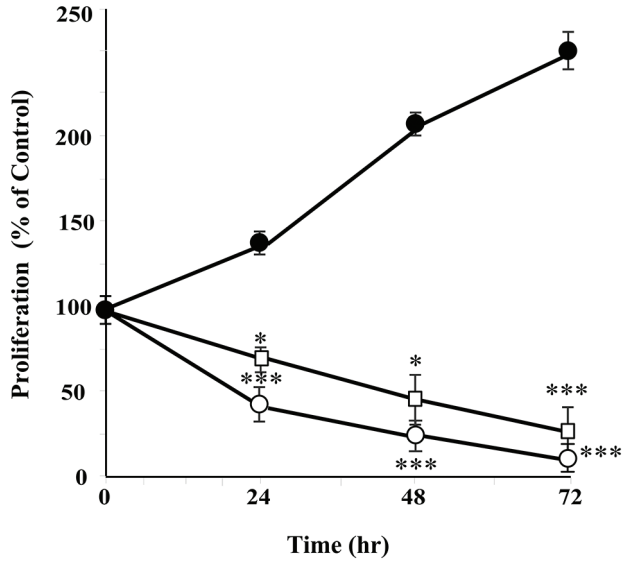

FIGURE 3. *Annona montana* hexane sub-fraction V suppressed cell proliferation. MDA-MB-231 cells were treated with 0.01% dimethyl sulfoxide (DMSO) alone (●), 20 μg/mL (□), and 40 μg/mL (○) of hexane sub-fraction V of *A. montana* several times (24, 48, and 72 h). Cell proliferation was quantified by spectrophotometric analysis using MTT assay as described in Material and Methods. Results were expressed as a percentage of 0.01% DMSO-treated cells (control cells). Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ as determined by two-way ANOVA followed by Tukey's *post hoc* test.

proliferation of MDA-MB-231 cells for up to 72 h. Specifically, MDA-MB-231 cells were incubated with 0.01% DMSO, 20 μg/mL, or 40 μg/mL of *A. montana* hexane extract for three different time intervals: 24, 48, and 72 h, respectively. After the last incubation, the cell proliferation was analyzed using an MTT assay described in Material and Methods. As shown in Fig. 3, 40 μg/mL of *A. montana* hexane subfraction V significantly suppressed cell proliferation of MDA-MB-231 cells in a time-dependent matter with potent inhibition of cell proliferation (>90% inhibition) at 72 h of treatment.

Anonna montana hexane subfraction V extract induced apoptosis in MDA-MB-231 breast cancer cells

To further define the inhibitory effect of *A. montana* hexane sub-fraction V, we investigate the effect of this extract on the cell cycle by flow cytometry as described in Material and Methods. In Figs. 4A and 4B, we show that after incubation of cells with 20 μg/mL *A. montana* hexane sub-fraction V for 48 h, a significant increase in the SubG1 peak (i.e., untreated cells: 3 ± 0.2; treated cells: 27 ± 2.1). Also, the percentage of cell population in the Go/G1, S, and G2+M phases was significantly lower than in untreated cells. Taken together, our data indicate that treatment of cells with *A. montana* hexane sub-fraction V was effective and led to the suppression of cell proliferation and induced apoptosis, as evidenced by the increased accumulation of cells in the Sub-G1 phase of the cell cycle. Based on our observations that continued the exposure of *A. montana* hexane sub-fraction V to MDA-MB-231 cells strongly diminished cell proliferation with an increase in the Sub-G1 peak, we decided to investigate the effect of this extract on apoptosis. We stained MDA-MB-231 cells with Annexin V-FITC after treating them with 20 μg/mL of *A. montana* hexane subfraction V for 24 h. Figs. 5A and 5B shows that cells stained with Annexin V antibodies (regarded as early apoptosis) increase compared to non-treated cells (Control). We then examined for late apoptosis using the TUNEL assay. Again, apoptotic cells were significantly increased on treatment with *A. montana* hexane subfraction V for 24 h, as observed by flow cytometry (compare Figs. 5C and 5D). In Table 3, we also show more specific results of Annexin V FITC and PI staining of MDA-MB-231 cells treated with 20 μg/mL of *A. montana* hexane subfraction V at two different times intervals in addition to 24 h intervals (i.e., 48 and 72 h). A significant increase in the population of early apoptotic cells from 1.14% (untreated cells) to 21.70%

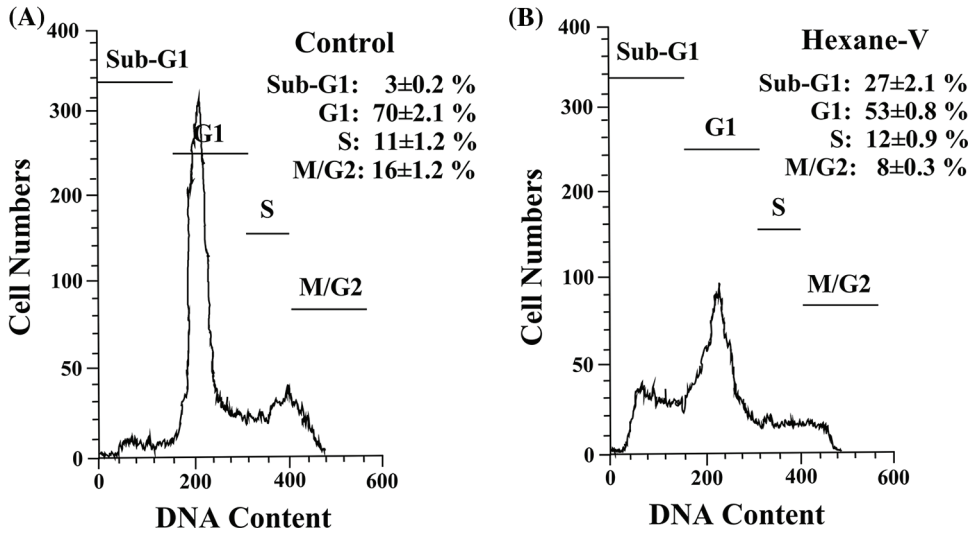

FIGURE 4. Histogram analysis of the cell cycle in MDA-MB-231 cells in the presence of *Annona montana* hexane subfraction. (A, B) MDA-MB-231 cells were treated with 0.01% DMSO alone (control) or 20 μg/mL of hexane sub-fraction V (hexane-V) of *A. montana* for 8 h. Then, the cells were fixed, stained with propidium iodide, and analyzed using a flow cytometer as described in the Material and Methods section. The data are representative of one of four independent experiments. Inset: Data represent the mean ± SEM of four independent experiments.

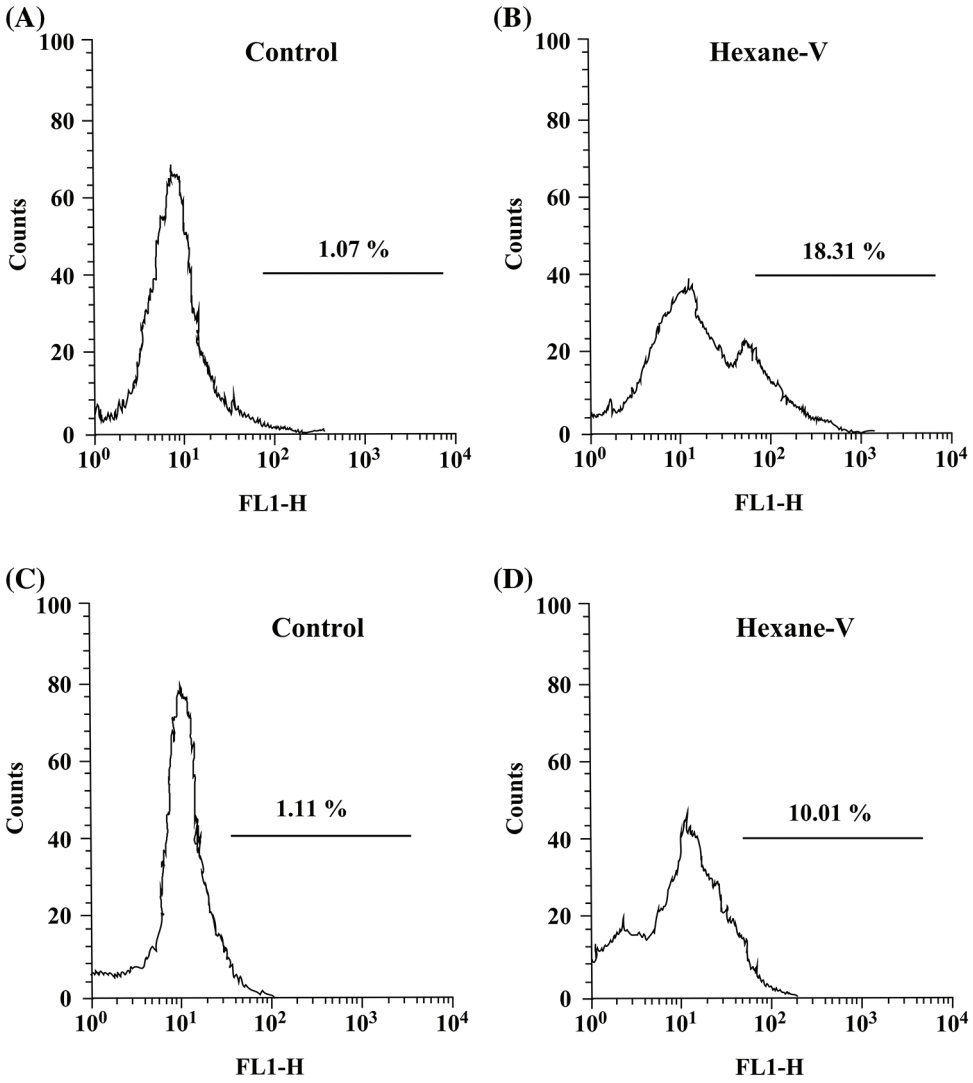

FIGURE 5. *Annona montana* hexane subfraction V induced apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were treated with 0.01% DMSO alone (control) or 20 µg/mL of hexane sub-fraction V (hexane-V) of *A. montana* for 24 h. Then, the cells were fixed, stained with a FITC-conjugated Annexin V antibody (A, B), incubated using TUNEL reaction solution (C, D), and then analyzed using a flow cytometer as described in Material and Methods. The data are representative of one of four independent experiments.

(treated cells) when cells were incubated with 20 µg/mL *A. montana* hexane subfraction V for 48 h. Moreover, a further increase in the population of early apoptotic MDA-MB-231 cells after 72 h of incubation (i.e., untreated cells: 3.09; treated cells: 37.01). Similarly, we also observed an increase in the population of late apoptosis at 48 h (i.e., untreated cells: 1.33; treated cells: 12.21) and 72 h (i.e., untreated cells: 1.39; treated cells: 15.31). After treatment, dead cells were also increased with 20 µg/mL *A. montana* hexane subfraction V (Table 3). These results suggest that *A.*

TABLE 3

The percentage of apoptotic cells post-treatment of *Anonna montana* hexane subfraction-V treatment at 24, 48, and 72 h were determined from annexin V/propidium iodide double staining

Treatment duration	Percentage of cells (%)			
	Viability	Early apoptosis	Late apoptosis	Dead
Control (24 h)	96.01 ± 1.9	1.07± 0.08	1.11 ± 0.02	1.23 ± 0.03
Hexane (24 h)	61.51 ± 2.5	18.31 ± 0.06*	10.01 ± 0.03*	1.31 ± 0.07
Control (48 h)	95.07 ± 3.1	1.14 ± 0.03	1.33 ± 0.04	1.61 ± 0.08
Hexane (48 h)	57.71 ± 2.5**	21.51 ± 0.05**	12.24 ± 0.07**	1.74 ± 0.01
Control (72 h)	94.23 ± 3.5	3.09 ± 0.05	1.39 ± 0.06	1.89 ± 0.03
Hexane (72 h)	49.31 ± 2.2***	37.01 ± 0.03***	15.31 ± 0.05***	2.01 ± 0.09

Note: Each data was expressed as mean ± standard error of triplicate determinations' the mean (SEM). *Statistical significance ($p < 0.05$) between control and hexane subfraction-V-treated group in 24-h time-point by student's *t*-test. **Statistical significance ($p < 0.01$) between control and hexane-treated group in 48-h time-point by student's *t*-test. ***Statistical significance ($p < 0.001$) between the control and hexane-treated group in 72-h by student's *t*-test.

montana hexane subfraction V arrested the cell cycle at the Sub-G0/G1 phase and induced apoptosis *in vitro* in a time-dependent manner.

The anti-metastatic potential of Anonna montana hexane subfraction V extract on MDA-MB-231 cells

The anti-metastatic abilities of the *A. montana* hexane subfraction V were evaluated in MDA-MB-231 cells by using several *in vitro* assays (i.e., wound healing analysis, migration, invasion, and clonogenic assay). In Fig. 6A, we show a significant decrease in the percentage of wound closure in the *A. montana* hexane subfraction V extract-treated MDA-MB-231 cells compared with untreated cells (compare 67 ± 4% of closure of treated cells *vs.* 100% closure for untreated-control cells) after 48 h incubation. We also observed that cells treated with *A. montana* hexane sub-fraction V inhibited migration (83 ± 7% inhibition) through the Transwell membrane compared to untreated cells (Fig. 6B). Then, we evaluated the invasive capacity of MDA-MB-231 cells in an *in vitro* system. From the Matrigel invasion assay, as shown in Fig. 6C, the ability of MDA-MB-231 cells to invade this loaded membrane was significantly compromised (72 ± 5% inhibition invasion) in the *A. montana* hexane sub-fraction V-treated cells relative to the control cells. We also investigated the effect of the *A. montana* hexane sub-fraction V on the ability of MDA-MB-231 breast cancer cells to form colonies to understand better the inhibitory effect of *A. montana* hexane sub-fraction V on MDA-MB-231 breast cancer cells. From the clonogenic assay, as shown in Fig. 6D, the ability of MDA-MB-231 cells to form colonies was significantly reduced (64 ± 5% inhibition of invasion) in the *A. montana* hexane sub-fraction V-treated cells relative to the control cells. In addition, the inhibitory effect of *A. montana* hexane subfraction V on all these assays was time and concentration-dependent (data not shown). Furthermore, we observed that the protein expressions of MMP-2 and MMP-9 were analyzed to evaluate the effect of *A. montana* hexane sub-fraction V on the motility of MDA-MB-231 cells. It showed that *A. montana* hexane sub-fraction V significantly suppressed the protein expressions of MMP-2 (84 ± 5% inhibition of protein expression) and MMP-9 (54 ± 3% inhibition of protein expression) (Fig. 6E). Thus, these results indicated that *A. montana* hexane sub-fraction V significantly inhibited the migration, invasion, and colony formation of MDA-MB-231 breast cancer cells *in vitro*. These observations are consistent with the fact that MMP-2 and MMP-9 proteins are closely related to the migration and invasion of various types of cancer cells (Brown and Murray, 2015; Folgueras *et al.*, 2004).

Anonna montana hexane subfraction V extract inhibits Ras signaling cascades

The Ras small GTPases control signaling pathways that are key regulators of several aspects of the uncontrol cell growth (Downward, 2003). The downstream of Ras proteins is the RAF/MEK/ERK kinase cascade (Kolch *et al.*, 1991). Also, the active Ras turn on the PI3K/AKT/mTOR/S6K1 pathway regulates different cellular functions (i.e., anti-apoptotic pathways) (Yuan *et al.*, 2020). We first investigated whether *A. montana* hexane sub-fraction V extract affects the PI3K/AKT/mTOR/S6K1 activation in MDA-MB-231 breast cancer cells. As shown in Figs. 7A–7D, PI3K and Akt activation was suppressed by 20 μg/mL of *A. montana* hexane subfraction V. In addition, the activation of S6K1 and mTOR was also decreased upon *A. montana* hexane extract treatment in MDA-MB-231 breast cancer cells. Our data indicate that inhibiting PI3K/AKT/mTOR/S6K1 signaling cascade by *A. montana* hexane sub-fraction V leads to the suppression of cell proliferation in MDA-MB-231 breast cancer cells. We next determine whether *A. montana* hexane subfraction V could alter the activation of MAPK cascades, including JNK, ERK1/2, and p38 MAPK in MDA-MB-231 breast cancer cells. As shown in Figs. 7E–7H, *A. montana* hexane subfraction V substantially decreased the phosphorylation of Raf-1 (84 ± 5% inhibition) and Erk1/2 (56 ± 3% inhibition) at a concentration of 20 μg/mL and slightly downregulated the phosphorylation of JNK (30 ± 4% inhibition) and p38 MAPK (19 ± 5% inhibition) in MDA-MB-231 breast cancer cells.

Anonna montana hexane subfraction V extract inhibits the expression of essential signaling proteins involved in apoptosis, proliferation, and angiogenesis

Our observations indicate that AKT and ERK1/2 activities are affected by the *A. montana* hexane subfraction V. We next examined the effects of *A. montana* hexane subfraction V on the expression of survivin, IAP-1, and IAP-2 proteins. They have been implicated in apoptosis and act downstream to PI3K/Akt/mTOR/S6K1 signaling cascade (Hassan *et al.*, 2013; Paplomata and O'Regan, 2014). We found that *A. montana* hexane subfraction V suppressed the expression of anti-apoptotic proteins (Figs. 8A–8C). Also, *A. montana* hexane sub-fraction V decreased the expression of Cyclin D1, COX-2, and VEGF (Figs. 8D–8F). In addition, we determined the effect of *A. montana* hexane sub-fraction V on the expression and cleaved procaspase-3 and PARP. Procaspase-3 is an excellent marker to monitor activated apoptotic and triggers cleavage of PARP, which is involved in repairing DNA damage (Kitazumi and Tsukahara, 2011). In Figs. 8G and 8H, we showed that *A. montana* hexane subfraction V induced the cleavage of procaspase-3 and PARP, as observed by the disappearance of the main band and the appearance of its cleaved forms. The tumor-suppressor proteins p53 and p21 are significant regulators of the cell–cycle checkpoint (Kulaberoglu *et al.*, 2016). The p53 protein is a tumor suppressor in preventing cancer development (Luo *et al.*, 1995). The cyclin-dependent kinase inhibitor p21 is regulated by p53 and negatively modulates cell cycle progression by inhibiting the activities of cyclin E/CDK2 and cyclin D/CDK4 complexes (Pandey *et al.*, 2019). We found that *A. montana* hexane subfraction V induced the expression of both p53 and p21 in MDA-MB-231 breast cancer cells (Figs. 8I and 8J). These results demonstrate that *A. montana* hexane subfraction V induces apoptosis via the down-regulation of anti-apoptotic, proliferative, metastatic, and angiogenetic proteins via caspase-3 activation in MDA-MB-231 breast cancer cells.

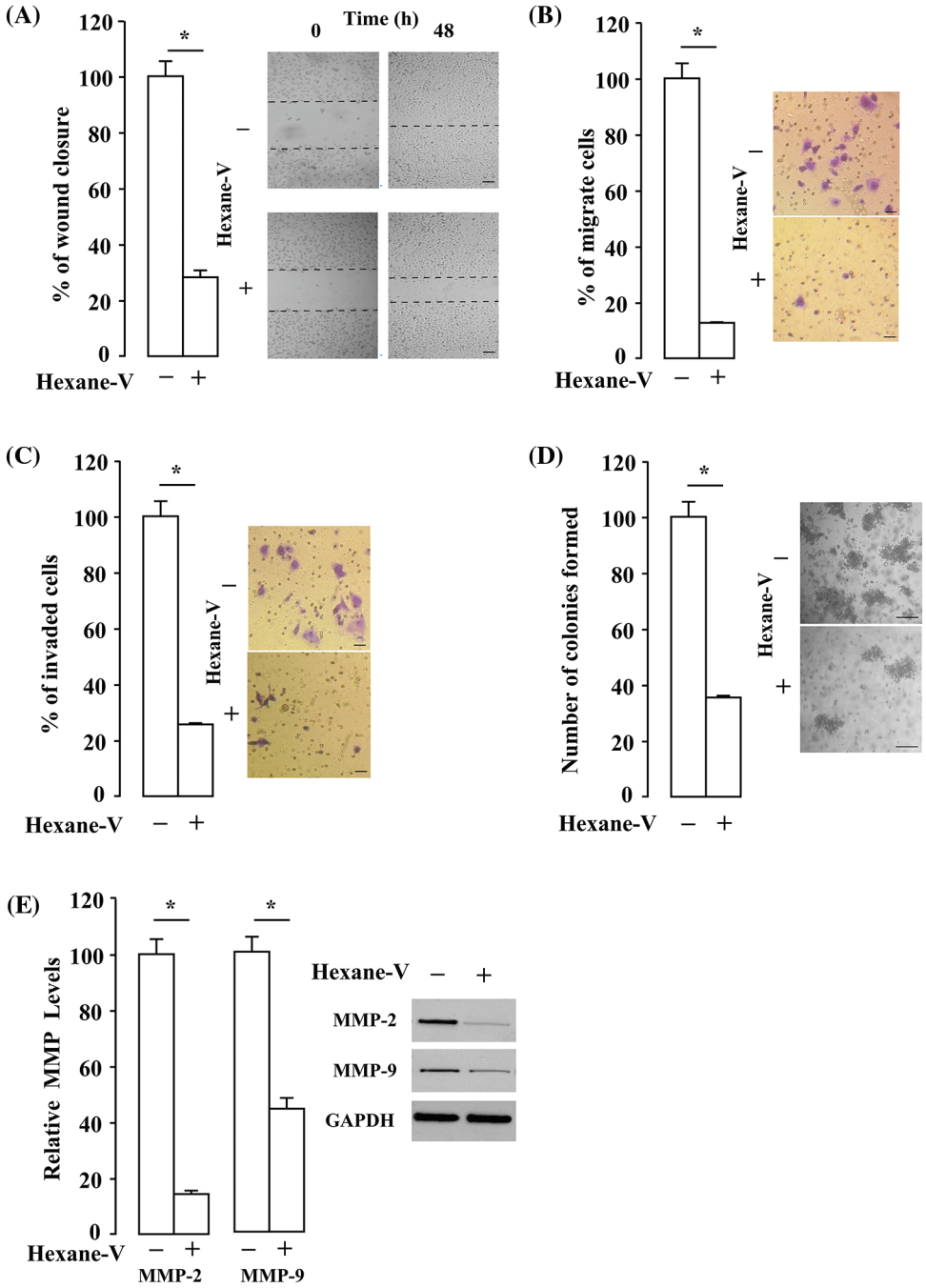

FIGURE 6. Effect of *Annona montana* hexane subfraction V on migration, invasion, and colony formation. MDA-MB-231 cell migration was analyzed by wound healing assay (scale bar is 200 μm) (A) or by Transwell invasion assay (scale bar is 50 μm) (B) in the presence of 0.1% dimethyl sulfoxide (DMSO) alone (control) or 20 μg/mL of hexane subfraction V (hexane-V) of *A. montana*. (C) MDA-MB-231 cell invasion was examined by Transwell invasion assay in the presence of 0.01% DMSO alone (control) or 20 μg/mL of hexane subfraction V (hexane-V) of *A.* montana (scale bar is 50 μm). (D) MDA-MB-231 cell colony formation was detected following culturing with 30 μg/mL *A. montana* hexane subfraction V (hexane-V) of *A. montana* for 14 days (scale bar is 100 μm). (E) The expression of MMP-2 and MMP-9 was analyzed by western blotting t using *A. montana* hexane subfraction V (hexane-V) for 8 h. Then, equal amounts of lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to a nitrocellulose membrane, and antibodies specific to MMP-2 and MMP-9 antibodies were used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Inset: the data are representative of one of four independent experiments. Western blotting was performed three times, and a representative image of the three independent experiments is shown. The results are expressed as the mean ± SEM of three independent experiments, and each was performed in triplicate. *$p < 0.05$ *vs.* DMSO-treated group (control). Hexane-V, *A. montana* hexane subfraction V; OD, optical density.

Anonna montana hexane subfraction V extract potentiates the inhibition of pharmacological blockers

To further understand how *A. montana* hexane subfraction V may modulate various signaling molecules downstream of Ras pathways, we decided to treat MDA-MB-231 breast cancer cells with the following inhibitors (i.e., PF-3758309, FR180204, Wortmannin, and ISIS2503). We then investigated whether the *A. montana* hexane subfraction V was able to enhance its inhibitory effect by combination with the pharmacological inhibitors mentioned above. As

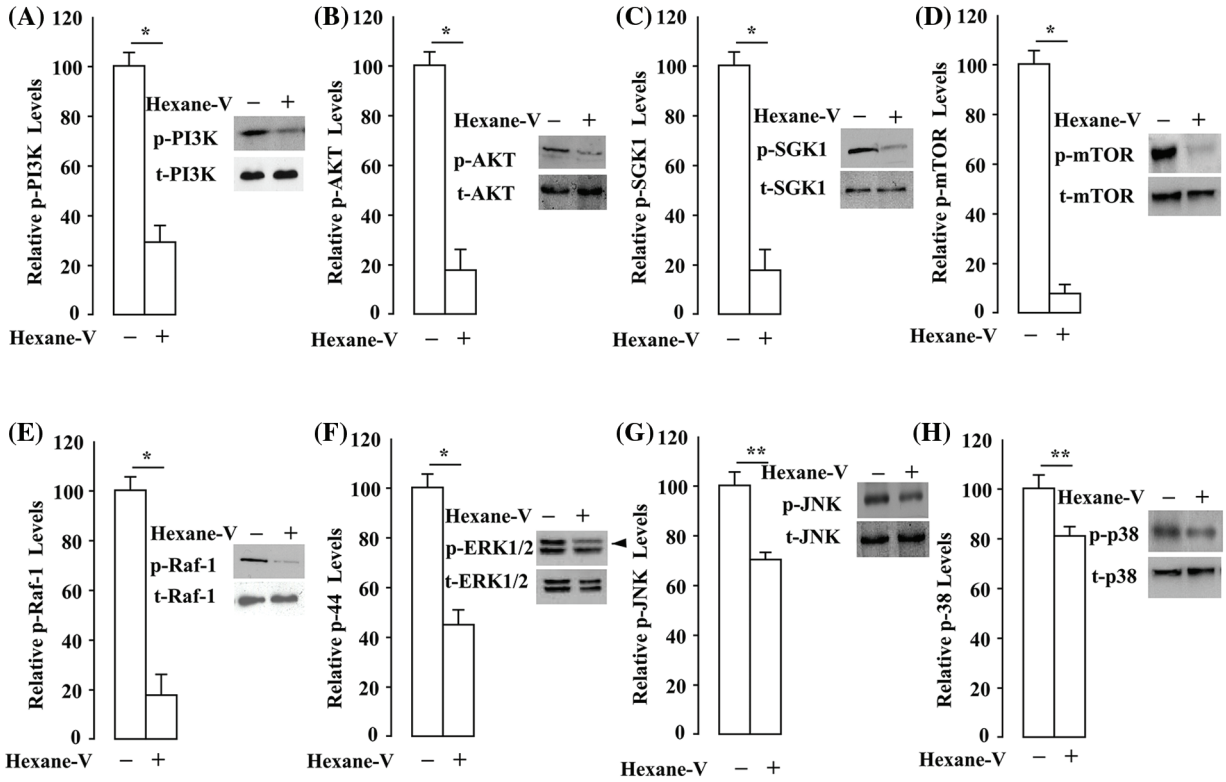

FIGURE 7. *Annona montana* hexane subfraction V suppressed PI3K/AKT/mTOR/S6K1 and MAPKs signaling pathways in breast cancer cells. (A–H) MDA-MB-231 cells were incubated with 0.01% DMSO or 20 μg/mL hexane subfraction of V (hexane-V) *A. montana* for 8 h as described in Material and Methods. Then, equal amounts of lysates were subset to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and antibodies specific to tyrosine-phosphorylated (p)-PI3K (Tyr458), PI3K, p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-S6K1 (Thr 421/Ser424), S6K1, p-ERK (Thr202/Tyr204), t-ERK, p-JNK (Thr183/Tyr185), JNK, p-p38 (Thr180/Tyr182), and p38. Relative levels of phosphorylated proteins were determined by densitometry as described in Material and Methods, and they were expressed as a ratio of phosphorylated/total proteins. The western blotting was performed three times, and a representative image of the three independent experiments is shown. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ by student's *t*-test compared to non-treated cells.

shown in Figs. 9A–9D, treatment of cells with either *A. montana* hexane subfraction V or wortmannin inhibited AKT activation but had a weak inhibitory effect on mTOR and S6K1 activation. Interestingly, mTOR and S6K1 activation were blocked by combining *A. montana* hexane subfraction V alone or with wortmannin. In Figs. 9E–9G, we showed that the PF-3758309, a PAK1 inhibitor, decreased the phosphorylation of RAF. Moreover, it also affected the activation of ERK1/2. However, the addition of FR180204, an ERK1/2 inhibitor, strongly diminished the activation of ERK1/2 without affecting the activity of RAF. Interestingly, we also observed inhibition of the phosphorylation of RAF and ERK1/2 when *A. montana* hexane subfraction V was combined individually with each inhibitor. We also observed that the addition of *A. montana* hexane subfraction V was unable to affect the expression of Ras when compared with the ISIS2503 inhibitor alone in MDA-MB-231 breast cancer cells. As expected, the combination of *A. montana* hexane subfraction V with ISIS2503 inhibitor did not potentiate the inhibitory effect of the ISIS2503 inhibitor alone (Figs. 9H and 9I). We next examined the effects of *A. montana* hexane subfraction V extract on the activation of AKT and ERK1/2 activities upon IGF-1 stimulation. Fig. 10 shows that AKT and ERK1/2 activities were affected by the *A. montana* hexane subfraction V upon IGF-1 stimulation. However, neither IGF-1 receptor phosphorylation nor activation of Ras was affected. These data imply that *A. montana* hexane subfraction V can act downstream of the Ras protein through modulation of multiple signal transduction pathways to induce apoptosis in tumor cells.

Chemical composition of the Anonna montana hexane subfraction V extract

Chemical studies with species of the Annona genus have reported on the essential oils from fruits because many fruits from the Annonaceae family are edible (Nugraha *et al.*, 2019; Al Kazman *et al.*, 2022). On the contrary, only some studies have been performed on the leaves. Thus, we conducted a GC-MS analysis as described in Material and Methods section to gain more information about the composition of the constituents, specifically in the *A. montana* hexane sub-fraction V. Our results on *A. montana* hexane sub-fraction V indicated that ten compounds out of a total of fifteen compounds were identified, (Table 4 and Suppl. Table S1) in a complex mixture of phytosterols

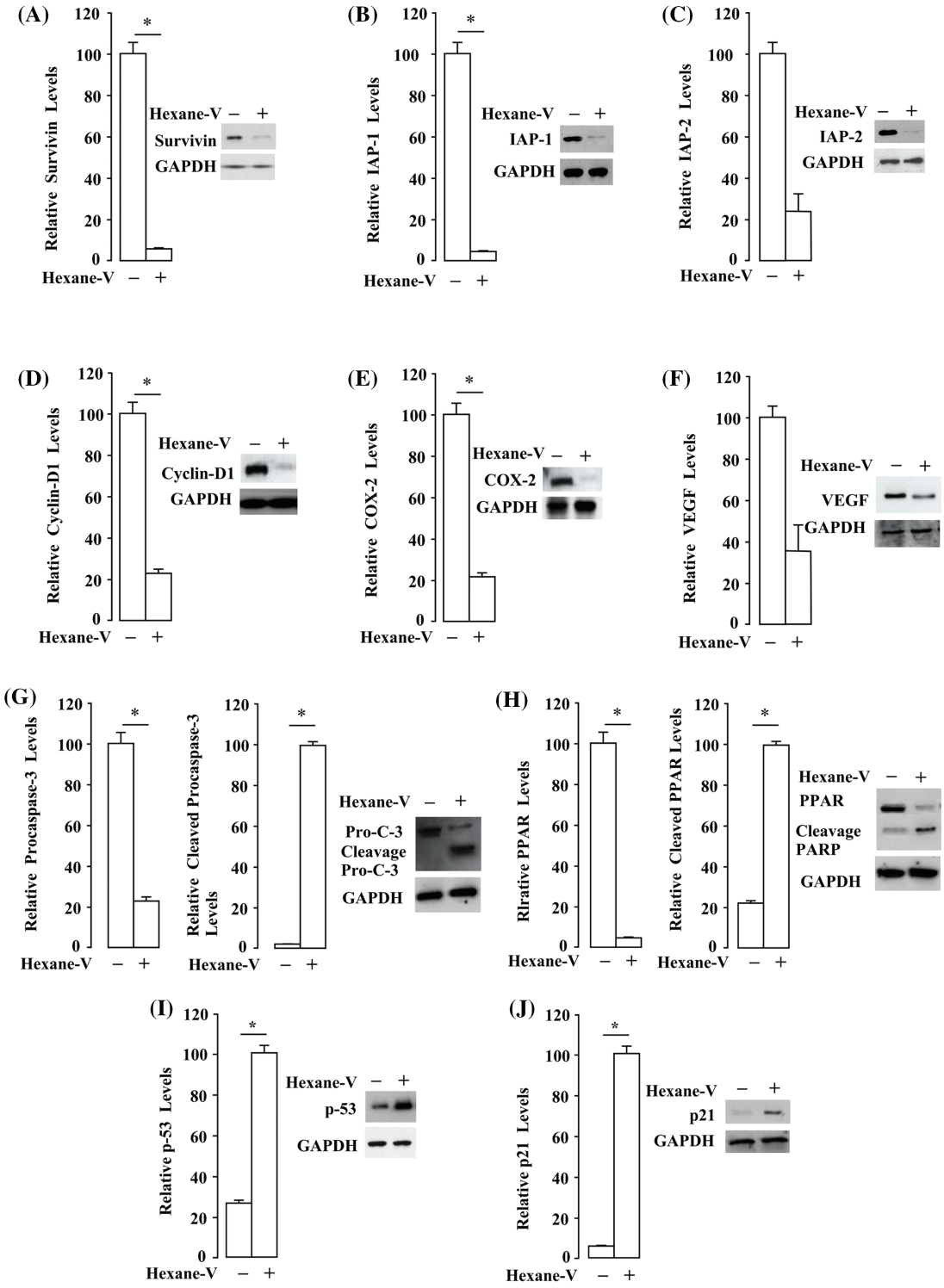

FIGURE 8. *Annona montana* hexane subfraction V decreases the expression of proteins involved in proliferation, anti-apoptotic, metastatic, and angiogenetic proteins. (A–H) MDA-MB-231 cells were treated with 0.01% DMSO or 20 μg/mL hexane subfraction (hexane-V) of *A. montana* for 8 h. Thereafter, equal amounts of lysates were analyzed by western blot analysis using antibodies against survivin, IAP-1, IAP-2, Cyclin D1, COX-2, VEGF, procaspase-3, cleaved caspase-3, and PARP. (I, J) MDA-MB-231 cells were treated with 0.01% DMSO or 20 μg/mL hexane subfraction V of *A. montana* for 8 h. After, equal amounts of lysates were analyzed by western blotting analysis using antibodies against p21 and p53. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Relative levels of each protein were determined by densitometry as described in the Material and Methods section, and they were expressed as a ratio of each protein/GAPDH. The data represents the mean ± SEM of three independent experiments. The western blots were performed three times, and a representative image of the three independent experiments is shown. *$p < 0.05$, and **$p < 0.01$.

(i.e., campesterol, γ-sitosterol, β-sitosterol, and γ-stigmasterol), γ-tocopherol, 1,2-epxyoctadecane, methyl-elaidolinolenate, 2-clhloroethyllinoleate, solanesol, and palmitic acid. Interestingly, some of these compounds have been reported to affect cell proliferation and apoptosis cellular mechanisms in cancer cells (Awad and Fink, 2000;

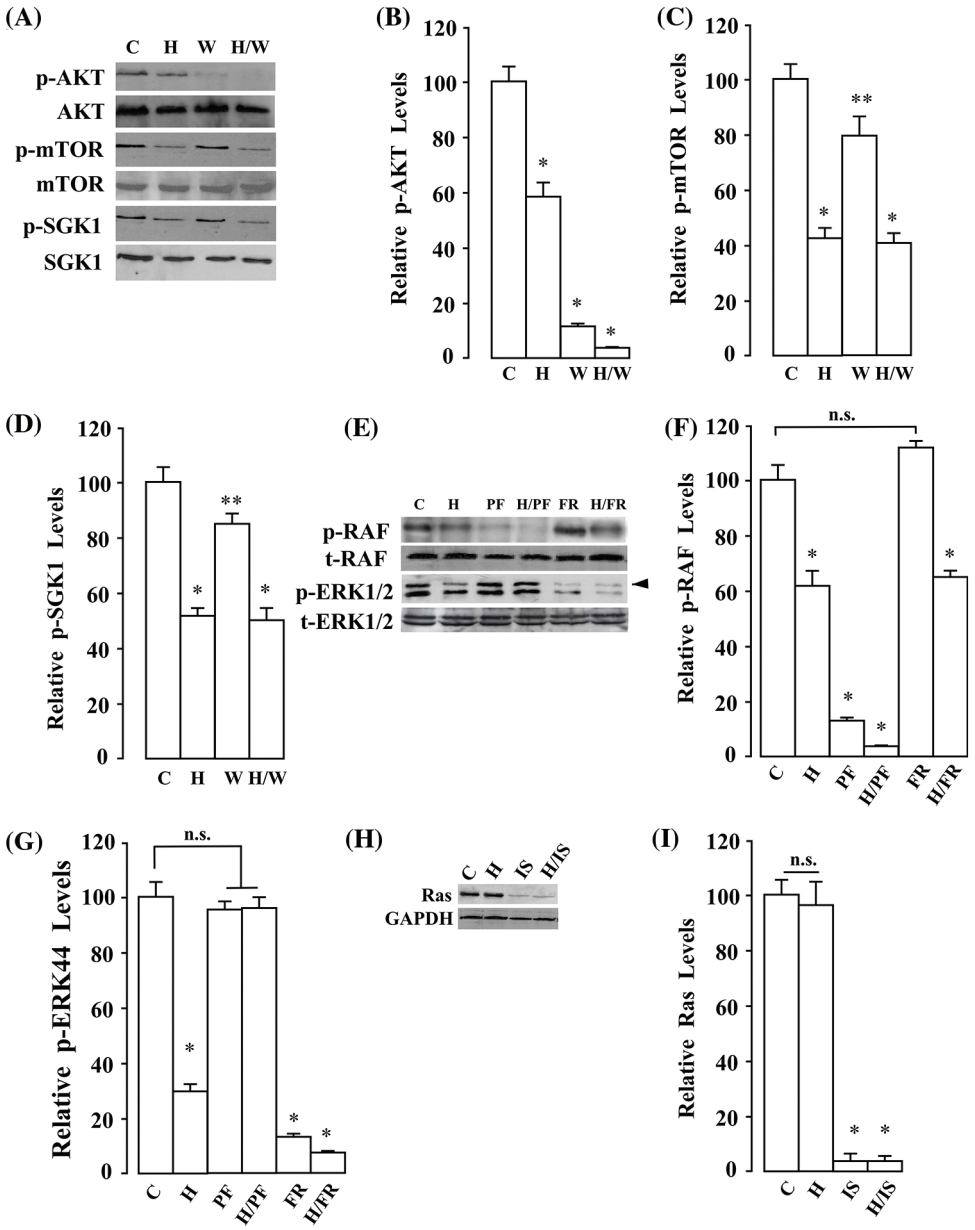

FIGURE 9. *Annona montana* hexane subfraction potentiated the apoptotic effects of pharmacological PI3K/AKT blockers and Ras inhibitors on the blockage of the Ras/RAF/ERK signaling pathway in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with 0.01% DMSO (Control) and the indicated combination of hexane sub-fraction V (H, 20 μg/mL) or wortmannin (W, 0.233 μM) for 8 h. Then, equal amounts of lysates were analyzed by western blotting using antibodies against p-AKT (Ser473), t-AKT, p-mTOR (Ser2448), t-mTOR, p-S6K1 (Thr421/Ser424), and t-S6K1. The western blots were performed three times, and a representative image of the three independent experiments is shown. (B–D) Relative levels of phosphorylated proteins (i.e., p-AKT, p-mTOR, p-S6K1) were determined by densitometry as described in the Material and Methods section. They were expressed as a ratio of phosphorylated/total proteins. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$, and $^{**}p < 0.01$ by student's *t*-test compared to non-treated cells. (E) MDA-MB-231 cells were treated with 0.01% dimethyl sulfoxide (control), and the indicated combination of hexane subfraction V (H, 20 μg/mL), PF3758309 (PF, 20 μM), or FR180204 (FR, 50 μM) for 8 h. Then equal amounts of lysates were analyzed by western blotting using antibodies against p-RAF (Ser259), t-RAF, p-ERK (Thr202/Tyr204), and t-ERK. The western blots were performed three times, and a representative image of the three independent experiments is shown. (F, G) Relative levels of phosphorylated proteins (i.e., p-RAF, p-ERK) were determined by densitometry as described in the Material and Methods section. They were expressed as a ratio of phosphorylated/total proteins. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$ by student's *t*-test compared to non-treated cells. (H) MDA-MB-231 cells were treated with the indicated combination of hexane subfraction V (H, 20 μg/mL) or Ras Inhibitor-ISIS 2503 (IS). Then, equal amounts of lysates were analyzed by western blotting using antibodies against Ras. Western blotting was performed three times, and a representative image of the three independent experiments is shown. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (I) Relative levels of Ras proteins were determined by densitometry as described in the Material and Methods section, and they were expressed as a ratio of Ras/GAPDH proteins. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$ by student's *t*-test compared to non-treated cells.

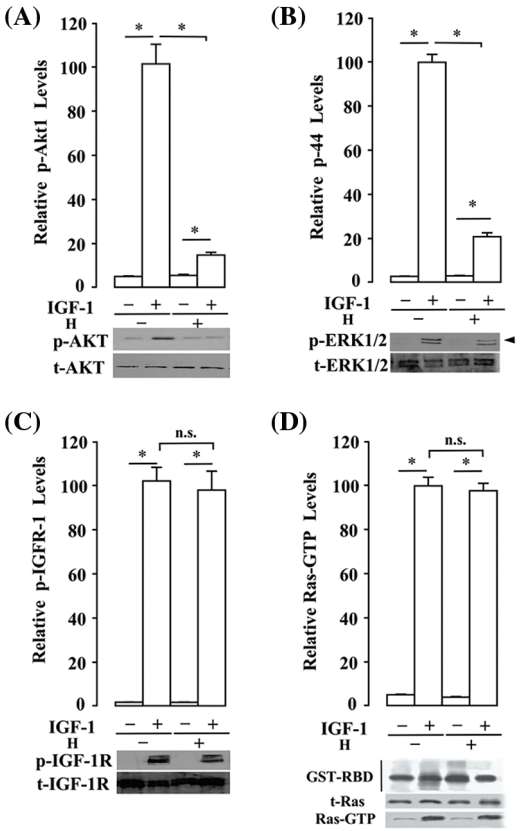

FIGURE 10. *Annona montana* hexane sub-fraction V suppressed AKT and ERK activities signaling in human breast cancer cells without affecting Ras activation upon IGF-1 stimulation. MDA-MB-231 cells were serum-starved for 16 h and then incubated in the presence of 0.01% dimethyl sulfoxide or 20 μg/mL hexane subfraction V (H) of *A. montana* for 8 h. Subsequently, cells were incubated in the presence of 2 ng/mL IGF-1 at 4°C for 90 min, washed with cold HBSS-BSA and incubated for 3 min at 37°C as described in the Material and Methods section. (A–C) Equal amounts of lysates were subset to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and antibodies specific to tyrosine-phosphorylated (p)-AKT (Ser473), total (t)-AKT, p-ERK (Thr202/Tyr204), t-ERK, p-IGF-1R (Tyr1131), and t-IGF-1R. Relative levels of phosphorylated proteins were determined by densitometry as described in the Material and Methods section, and they were expressed as a ratio of phosphorylated/total proteins. (D) After incubation, cells were washed with cold HBSS-BSA, lysed, and allowed to bind to glutathione beads in the presence of GST-RBD at 4°C for 60 min. Beads were washed with cold HBSS-BSA, and the presence of activated Ras (i.e., GTP-Ras) was analyzed by western blotting. Added GST-RBD and t-Ras proteins in the cell lysate were also evaluated by western blotting analysis. The Data represent the mean ± SEM of three independent experiments. $^*p < 0.05$ by student's *t*-test compared to non-treated cells.

Nugraha *et al.*, 2019; Al Kazman *et al.*, 2022; Cioccoloni *et al.*, 2022).

Discussion

Natural products from a variety of sources have been investigated to target several diseases, including cancer, for many years due to their biological activities and potential medicinal values contained in them. In this study, the anti-proliferative effect of the hexane leaf extract of *A. montana* was evaluated on breast cancer cell lines and other cancer cell lines with several methodologies. This hexane extract (i.e., *A. montana* hexane sub-fraction V) was found to contain a variety of bioactive compounds, including Campesterol, γ-Sitosterol, β-Sitosterol, and γ-Stigmasterol, which could explain the efficient and selective anti-breast cancer activity against triple-negative breast cancer cells (e.g., MDA-MB-231) and other cancer cell lines. Consistent with earlier findings (Leung *et al.*, 2022), *A. montana* extracts inhibited the differentiation of both mouse and human pre-adipocytes without any toxic effect by blocking the early steps of the differentiation process. Based on the anti-proliferative profile obtained, the hexane leaf sub-fraction V of *A. montana* was more selective towards MCF-7 than the MDA-MB-231 cell line. The proliferation of the HeLa cell line was also inhibited by adding *A. montana* hexane sub-fraction V. However, the treatment with the same subfraction affected the proliferation of A459 and Sk-MEL-28 less (Table 2). The anti-proliferative effect of primary mammalian epithelial cells and MCF-10A cell line in the presence of *A. montana* hexane extract was determined. It appeared that adding *A. montana* hexane extract was significantly less effective for normal cells as it required a higher dosage to kill cancer cells, suggesting the low side effect of this *A. montana* hexane extract. Together, these results indicate the selective effect of *A. montana* hexane extract in affecting cancerous cells with no damage to normal healthy cells. Several investigations on other related Annona species, like *A. muricata* and *A. squamosa*, have concentrated on their anti-proliferative effect against

TABLE 4

Chemical composition and percentage of compounds from the leaves of *Annona montana* hexane subfraction V

		RT/min	Area
1	Unknown	15.03	5.9%
2	Unknown	24.82	2.1%
3	Palmitic acid	15.03	1.6%
4	2-Chloroethyl Linoleate	31.09	3.1%
5	Methyl elaidolinolenate	31.36	4.6%
6	Unknown	33.03	18.7%
7	1,2 epoxyoctadecane	41.8	1.2%
8	γ-Tocopherol	44.34	6.6%
9	Unknown	46.13	1.1%
10	Campesterol.	47.27	10.1%
11	β-Sitosterol	47.61	3.9%
12	Unknown	48.12	10.1%
13	β-Stigmasterol	48.68	15.3%
14	γ-Sitosterol	48.32	10.1%
15	Solanesol	51.92	15.3%
		Total	**93.2%**

Note: RT: Retention time. The compounds were identified using the National Institute of Standards and Technology (NIST) EPA NIH mass spectral database program (NIST v17; version 2.3).

different cancer cell lines (Wang *et al*., 2014; Syed Najmuddin *et al*., 2016). Other studies revealed the anti-proliferative effect of *A. cherimola* leaves and seeds on cancer and leukemic cells (Arunjyothi *et al*., 2012; Suresh *et al*., 2011; Haykal *et al*., 2019). Recent studies revealed the anti-proliferative effect of *A. montana* fruit on several cancer cell lines (Bailon-Moscoso *et al*., 2016). Here, we observed that the hexane subfraction V of *A. montana* exerted significant anti-proliferative and apoptotic effects and suppressed the PI3K/AKT/mTOR/S6K1 activation and in less extent, the MAPKs activation in human breast cancer cells. Consequently, this *A. montana* hexane sub-fraction V down-regulated various gene products involved in cell migration, proliferation, apoptosis, and invasion. Thus, the treatment of cancer cells with *A. montana* hexane sub-fraction V targeted more selectively the PI3K/AKT/mTOR/S6K1 more than the MAPK signaling pathways and, more critical, induced apoptosis through caspase-3 activation. We found that *A. montana* hexane subfraction V blocked proliferation and the expression of various genes involved in the cell cycle. The cyclin D1, as well as p70S6K, are required for cell growth and the cell cycle progression in tumor cells (Mita *et al*., 2003). In addition, we also found that the expression of p53 and p21 tumor suppressors were upregulated upon Am hexane sub-fraction V treatment. These tumor suppressors can regulate the activities of cyclin-dependent kinase complexes, were also upregulated upon *A. montana* hexane sub-fraction V treatment. We also observed that *A. montana* hexane sub-fraction V could block surviving expression, suggesting that the use of *A. montana* hexane sub-fraction V can lead to the development of a novel strategy to regulate the stimulatory effect of IGF-1 on the proliferation of breast cancer cells (Costa-Silva *et al*., 2020). Interestingly, we also found that either the expression and activation of IGF-1R and Ras were not affected, suggesting a selective effect of *A. montana* hexane sub-fraction V on these signaling pathways. Furthermore, we found that this *A. montana* hexane sub-fraction V can also significantly potentiate the apoptotic effects of specific AKT inhibitors. This potentiation is likely mediated by suppressing anti-apoptosis genes regulated by the PI3K/mTOR pathways. Our results also indicate, for the first time, that *A. montana* hexane sub-fraction V suppressed constitutive mTOR (Ser2448) activation in breast cancer cells. Recently, several mTOR inhibitors have shown great promise in clinical trials for treating various malignant tumors, where activation of mTOR is closely associated with tumorigenesis (Gentric *et al*., 2017; Ciolczyk-Wierzbicka *et al*., 2020; Xu *et al*., 2020; Popova and Jucker, 2021). Thus, it is possible that targeting the mTOR signaling pathway with a variety of compounds from natural sources can reveal a number of potential candidates for the prevention and treatment of cancer. Consistent with this observation, we also found that *A. montana* hexane sub-fraction V blocked the expression of MMP-1 and MMP-9, vital tissue remodeling enzymes with multiple overlapping activities critical for wound healing, invasion, migration, and tumor progression *in vivo* (Hingorani *et al*., 2018). We additionally discovered that the expression of various genes involved in tumor initiation and promotion was also repressed by *A. montana* hexane sub-fraction V. These include proliferative (cyclin D1), anti-apoptotic (survivin, IAP-1, and IAP-2), metastatic (COX-2), and angiogenic (VEGF) gene products. AKT signaling through mTOR and MAPK activation in tumor cells are essential mechanisms of oncogenesis that can shield cancer cells from apoptosis and drug resistance *in vivo* (Populo *et al*., 2012). Thus, suppressing COX-2 and VEGF may be the potential link for inhibiting metastasis, angiogenesis, and invasion by *A. montana* hexane sub-fraction V.

Conclusion

Our results indicate for the first time that *A. montana* hexane sub-fraction V can block constitutive PI3K/mTOR signaling transduction pathways stimulate MAPKs activation, and lead to apoptosis induction by decreasing gene expression, facilitating tumor cell survival, metastasis, and angiogenesis in human breast cancer cells. This selective effect on signaling transduction pathways of the *A. montana* hexane sub-fraction V could be attributed to one or more compounds identified in this subfraction. Nevertheless, a further chemical and cellular evaluation of *A. montana* hexane sub-fraction V is needed to learn about its anti-proliferative mechanism.

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Availability of Data and Materials: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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Supplementary Materials

TABLE S1

Chemical composition and percentage of compounds from the leaves of *Annona montana* hexane subfraction V

Component	%	Ri[a]	Ri[b]	Identification[c]
Palmitic acid	1.6%	1978	1984	1,2
2-Chloroethyl linoleate	3.1%	2100	2092	1,2,3
Methyl elaidolinolenate	4.6%	2081	2086	1,2
1,2 Epoxyoctadecane	1.2%	1900	1901	1,2,3
γ-Tocopherol	6.6%	2987	2980	1,2,3
Campesterol	10.1%	3305	3354	1,2,3
β-Sitosterol	3.9%	3168	3167	1,2,3
β-Stigmasterol	15.3%	3238	3290	1,2
γ-Sitosterol	7.7%	3290	3351	1,2,3
Solanesol	1.2%	2132	2138	2,3

Note: Ri[a]: retention indices (DB-5 column) calibrated against n-alkanes; Ri[b]: reference retention indices recorded in the literature (Adams, 2007; Araujo *et al.*, 2007; Babushok *et al.*, 2011); I[c] 1 = Kovats retention index, 2 = mass spectrum, 3 = co-injection with authentic compound.