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Full Length Research Paper

Effect of *Mansoa alliacea* (Bignonaceae) leaf extract on embryonic and tumorigenic mouse cell lines

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***Mansoa alliacea* contains high concentrations of chemicals thought to be associated with the suppression of tumor growth. Additionally, this plant has been reported to possess analgesic, anti-fungal, and anti-bacterial properties, thereby providing other potential benefits for cancer patients. Low doses of a water extract of *M. alliacea* were applied to a cancerous and non-cancerous cell line. Doses between 1.254 to 10.04 mg/ml of extract applied to T3-HA cancer cells inhibited cell growth, but higher doses of 29.92 to 89.6 mg/ml destroyed colonies of the cancer cells. Application of the extract to NIH Swiss mouse cell cultures resulted in the inhibition of growth at higher concentrations, but at a concentration of 10.14 mg/ml, cell growth began to increase after three days. However, cell death was less at lower concentrations than that of T3-HA cancer cells, thereby confirming that lower concentrations of Ajo de Monte will inhibit cancer cell growth as well as initially inhibit non-cancer cells. Thus, *M. alliacea* extract selectively targets T3-HA mouse cancer cells but not NIH Swiss embryonic mouse cells. Future research may consider the use of this plant for human cancer patients.**

Key words: *Mansoa alliacea*, Garlic Vine, Ajo de Monte, cancer

INTRODUCTION

In the Amazonian jungles of east Ecuador, native people profess that many plants have healing or other beneficial properties. In Ecuador, indigenous people utilize a woody vine called "Ajo de Monte" or "Garlic Vine" to treat and alleviate a variety of ailments. Ajo de Monte is used as a seasoning in cooking and the leaves are used as a topical anesthetic by placing a leaf in contact with the patient's skin. The analgesic effect penetrates sufficiently to alleviate joint pain. A tea preparation of the leaves may be used to effect a systemic analgesic response. This systemic effect is regarded by the Waorani people in Ecuador to temporarily assuage musculoskeletal pain from over exertion (personal observation).

The Ajo de Monte woody vine produces net-branching leaves that when mature, average between 10 and 22.5 cm in length. New shoot growth originates in apical meristems from between two mature leaves. When mature, the plants produce purple flowers with a white

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center that fade to paler shades. Ajo de Monte is native to Brazil, Costa Rica, Ecuador, French Guyana, Guyana, Peru and Suriname (Taylor, 2006).

Taylor (2006) described Ajo de Monte as *Mansoa alliacea* and placed it in the family Bignoniaceae. Ajo de Monte is otherwise referred to as *Adenocalymma alliaceum* and *Bignonia alliaceae* (Rana et al., 1999; Pandya et al., 2012; Granados-Echegoyen et al., 2014). Herbal applications may use the name Ajos Sacha. Indigenous people of the Amazon have used Ajo de Monte for spiritual rituals and as a cure for medical maladies including "bumps, swellings, rheumatism, arthritis, colds, uterine disorders, inflammation, epilepsy, and infertility (Taylor, 2006). Other systemic problems such as fever, flu, body aches, cramps, fatigue, and pain are also treated (Zoghbi et al., 2009). In addition, the leaves may be applied in direct contact with the skin for analgesic purposes or are prepared instead as an infusion or decoction. Bark is prepared as a tincture or decoction, and the root is used as a tincture or cold maceration (Taylor, 1996). Although these ethnobotanical uses are common among indigenous populations living within the natural range of *M. alliacea*, further study is necessary to confirm the clinical effectiveness of Ajo de Monte.

MATERIALS AND METHODS

Extraction

A leaf extraction was prepared using an adaptation of the procedure described in Rana et al. (1999) to produce a polar molecule extraction which could be applied as a component of cell growth medium. The effects of different concentrations of *M. alliacea* on the T3-HA mouse tumor cell line and normal NIH Swiss mouse cells were examined using a photomicrograhic method to evaluate *in vitro* cell growth. *M. alliacea* sample was obtained as a dry powder extract from Rain Tree Pharmaceuticals, a company avowing their support of sustainable harvesting of materials and rainforest preservation (Taylor, 1996, Rain Tree Pharmaceuticals, Milam County, TX). Fifty grams (50 g) *M. alliacea* powder was mixed with 250 ml water at 25°C and stirred for five min at room temperature. This 1 g/ 5 ml H_2O mixture formed a viscous liquid which was strained with cheesecloth. The effluent liquid was filtered via Buchner funnel suction filtration with #5 Whatman filtration paper. The resulting solution was centrifuged at 12,000 rpm for 20 min to eliminate chloroplasts and other organelles (Damon/IEC Division IEC HN-SII Centrifuge). The supernatant from centrifugation was then eluted through a 0.2 µm millipore filter. These sterile aliquots were frozen at -20°C for later use.

Cell culture

NIH Swiss mouse embryonic cells

Normal primary NIH Swiss mouse embryonic cells and the tumorigenic mouse T3-HA cell lines were utilized in this experiment. These cells were kindly provided by Durwood B. Ray and their origins, growth properties, morphologies, and tumorigenic capabilities have been described previously. The following description of cell lines and methodology was based on Ray et al. (2015). The NIH Swiss mouse embryonic non-transformed cells were produced as described by Todaro and Green (1963) by establishing primary cultures from eight whole 17 to 19 day old NIH Swiss mouse embryos. These normal mouse cells do not produce tumors when injected into mice and these normal cells exhibited a 22 h doubling time in previous assessment. The NIH Swiss embryonic cells used in this study were comprised of normal immortal cells within passage numbers from 10 to 22.

T3-HA tumor mouse cell line

The highly metastatic T3-HA tumor mouse cell line was obtained during the establishment of a new series of cell lines. They were derived from a new human h-ras oncogene (HRAS) transfection system using T24 human bladder carcinoma DNA to establish cells derived from it that represent various stages in one tumor progression that includes several metastatic cell types. The advantage of this approach is that all transformed cells in the series are derived from a common transfected parent population, namely, the GhrasT-NIH/3T3 cell line generated by transfection of immortalized NIH/3T3 cells with DNA from the T24 human bladder carcinoma. The design to intentionally expose different metastatic cells to *in* vivo micro-environmental effects as they adapt to multiple targets was accomplished by alternating between *in vitro* and *in vivo* growth in the development of this series. The first cell line in the series (T1-A) was cultured from a primary tumor in a NIH/Swiss mouse injected s.c. with the GhrasT-NIH/3T3 cells. The second cell line (T-2A) was cultured from a subsequent secondary local metastasis in a NIH/Swiss mouse injected i.v. with T1-A cells. The third cell line produced (T3-HA)(H=hepatic) used in this current study with *M. alliacea* extract was cultured from a tertiary liver metastatic tumor in a nude NIH/Swiss mouse injected i.v. with T2-A cells. Further cell line developments revealed the maintenance of the HRAS oncogene past the T3-HA stage. The tumorigenic growth and morphological properties of these newly reported T24 bladder carcinoma derived cell lines appear to be unique from those reported by others using a different human EJ-6-2-Bam-6a cell line. These outcomes indicated that the lung and liver are the most common sites for distant metastasis to occur in NIH Swiss nude mice injected i.v. in the tail vein with either T2-A or T3-HA cells. T3-HA cells exhibited a 17.5 h doubling time in previous assessment. The embryonic mouse cells and all the cell lines including the T3-HA cells were expanded and subcultures were frozen in Recovery Cell Culture Freezing Media (catalogue #12648-010, GIBCO) and stored in liquid nitrogen tanks as stocks.

Cell growth conditions

Embryonic mouse cells and T3-HA cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Catalog # 12320-032, GIBCO, Grand Island, NY) with extra added D (+) glucose (Catalogue # G-5400, Sigma Chemical Company, St. Louis, MO) (4.5 g/L), 10% fetal calf serum (Catalogue # SH30070.02, Hyclone Laboratories, Logan, Utah), and 20 units/ml of penicillin, 20 µg/ml streptomycin (Catalogue #15140-122, Sigma Chemical Company). Each culture was incubated at 37°C in 5% CO_2/95% air.

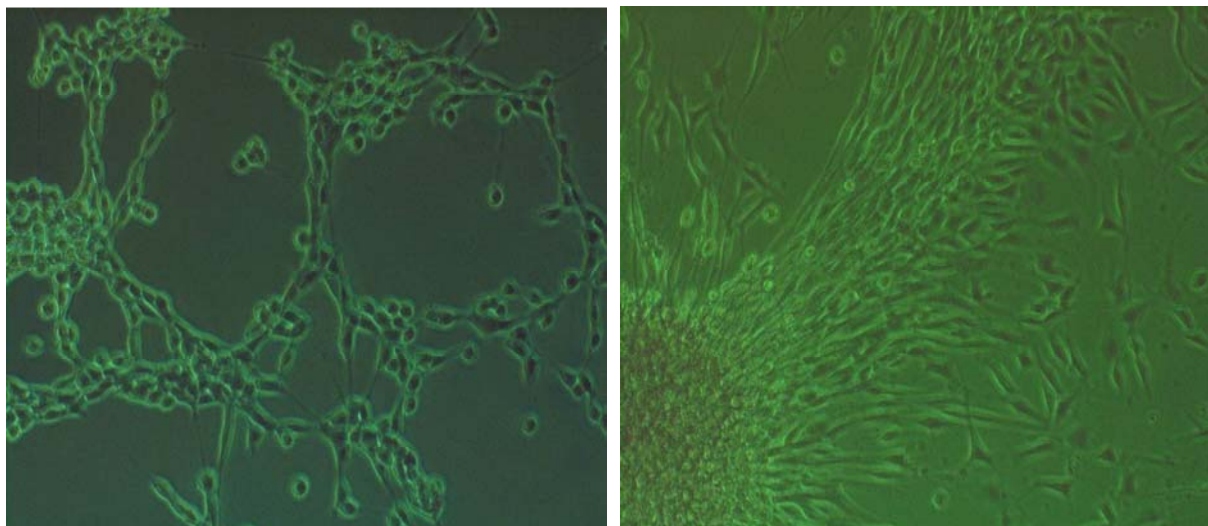

Figure 1. Growth comparison. A comparison of quantity of cultured T3-HA cells from Experiment 1 receiving little treatment over five days; cells from day one shown on left and day five shown on right.

Cell growth determination

A sterile cell culture protocol was utilized for all cell cultures. T3-HA cultured cancer cells or NIH Swiss mouse embryonic cells were plated onto 60 mm gridded tissue culture plates (Corning cat# 430196, Corning, NY). Six predetermined squares were pre-marked with marking pen on each dish prior to use. The squares were scattered in such a way that each 2 × 2 mm square area would be representative of the entire plate. Prior to each experiment, cells were plated and grown for 3 days prior to application of leaf extract. As a control solution, 900 µl H_2O was combined with 10 ml DMEM as described. The rapidly growing tumor cells used in this experiment metabolized the extra added glucose via aerobic glycolysis as their primary energy source and glycolytic intermediates (Warburg, 1956; Wallace et al., 2010). For the test solution, an aliquot of extract was thawed at room temperature, and 900 µl of Ajo de Monte (ADM) extract and 10 ml of media were mixed together within test tubes. Once the confluency of the cancer cells were assessed, the old media was aspirated off, and the plates were washed with sterile saline. Leaf extract was dissolved into cell culture media and pipetted onto each of the plates of cells to make a range of five different concentrations on a logarithmic scale as depicted in Table 1. The control plate of cells received only the growth media and water. Each of the plates received equivalent volumes of media and were swirled gently to distribute the solutions evenly over the plates.

At the same time each day for five days, six pictures were taken of each plate in specific 2 × 2 mm grids on each of the cell plates. Photomicrographs were taken daily when cells reached approximately 15 to 25% confluency (day 0). Photomicrographs were taken with an Olympus DP12 inverted microscope (Olympus America, Inc., Melville, NY) with a digital camera system at 100× in a standardized location within each selected 2 × 2 mm square. These photomicrographs were used to assess quantities of live cells once per day at the same time each day for five days by manual counting. Effectiveness of concentrations of Ajo de Monte extract were determined by observing cell death. Cell death is associated with cell detachment from the bottom of the culture plate. Remaining attached live cells can be easily counted.

Data analysis

The number of live cells was determined by manually counting cells in photomicrographs and subtracting the numbers of detached cells from the number of cells counted at the time the photograph was taken. Unattached cells were presumed to be dead and thereby not included in the cell counts. Counts were made on six 2 × 2 mm sampling areas on each plate and averaged for each day. Results for each day were normalized to fold increase relative to day 0. These procedures were used for the three treatments involving cancer cells and the two experiments using non-cancer cells. Normalized cell counts were produced by dividing the quantity of cells on a given day by the initial quantity of cells in the first photograph. The normalized cell count was used to compare populations of cells between plates. The mean normalized cell counts in all replications were averaged to compare the effects of different concentrations of Ajo de Monte extract between cancer and non-cancerous cell cultures. The differences in cell counts after the applications of Ajo de Monte extract were determined by comparing the average number of cell deaths per plate over five days of treatment as exemplified in Figure 1. The changes in the cell deaths were compared using analysis of variance (ANOVA) with an alpha value of 0.05 as significant for all treatments. Three replications were completed for treatments of T3-HA cancer cells and two replications for each treatment of NIH Swiss non-cancer cells.

A series of three experiments with identical concentrations of leaf extract was conducted with T3-HA mouse embryonic cells. Cell counts were compared before and after the trial and results across the series were averaged for each concentration. Concentration level was determined by grams dry weight leaf powder/ml cell culture media. This series produced growth curves depicted in Figure 2. Two experiments were conducted with NIH Swiss non-

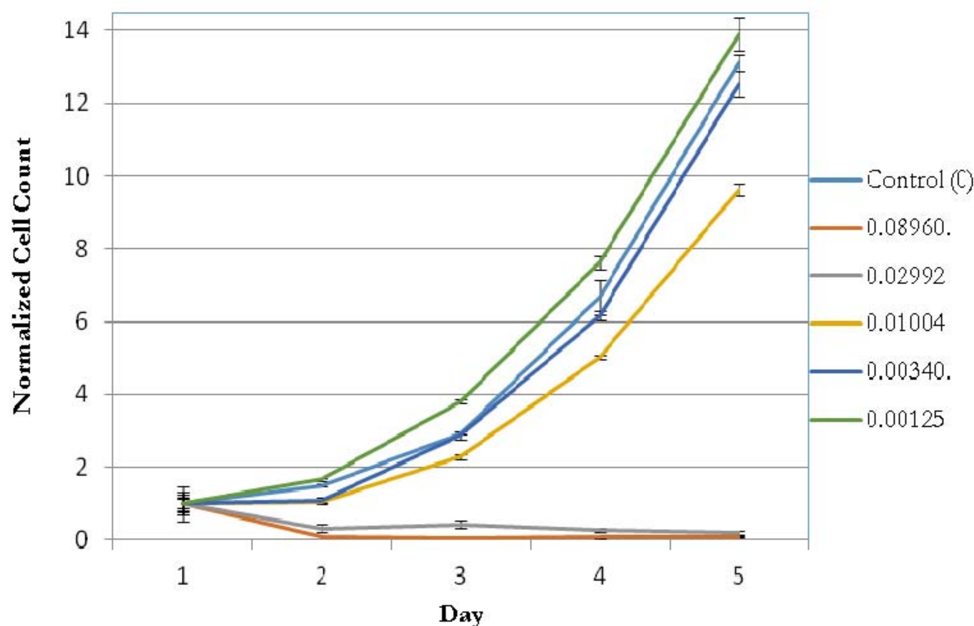

Figure 2. T3-HA Growth, mean normalized cell count of T3-HA cancer cells each day for each treatment condition (0 to 0.0896 g/ml) of *M. alliacea* extract. Normalized cell count produced by dividing the quantity of cells on a given day by the initial quantity of cells. “Control (0)” implies that water was added to the medium with no extract. Error bars represent the standard deviation.

cancerous cells under identical conditions as the experiments conducted with T3-HA cells. These data were averaged in the same way to produce Figure 3. NIH Swiss mouse cells were plated and treated using the same methods described for the T3-HA cancer cell line.

RESULTS

The normalized cell count was used to compare the final counts of cells with the initial cell counts for each experiment (Figures 2 and 3). There was a significant decline in the number of cancer cells at the treatment concentrations, whereas there was not a reduction in cancer cells at the lower concentrations (Figure 2). Moreover, the extract produced statistically different outcomes on the control (NIH Swiss, non-cancerous) populations of cells, as shown in Table 2. Figure 4 provides a visual of this effect. Garlic vine extract resulted in inhibition of the non-cancerous NIH Swiss cell growth without eliminating these populations or significantly reducing their population size. The two highest treatment levels revealed no significant difference in effect between themselves (sig ≤ 0.001). Most notably, at these levels of treatment, the cancerous cell population is significantly decreased but non-cancerous cells only are inhibited in their growth (sig ≤ 0.001).

DISCUSSION

Several researchers have studied other properties of Ajo de Monte, confirming fungicidal, anti-bacterial, cholesterol -lowering benefits, and anti-inflammatory characteristics (Pandya et al., 2012). Rana et al. (1999) reported that the exposure of *Alterniaria brassicae (Berk.) Sacc.* spores to an isolate from Ajo de Monte inhibited germination rates of the spores. Additionally, Khurana and Bhargava (1969) found that Ajo de Monte extract was effective against mild mosaic papaya viruses, whereas it was only slightly effective (20%) in eliminating distortion ring-spot and ring-spot viruses. Additional research is required to determine the efficacy of Ajo de Monte in other applications.

Ajo de Monte is seen as a possible treatment for cancer based on the alleged success in treating cancer patients in the town of Puyo, Ecuador (personal communication with Galo Ortiz, director of the Indigenous People’s Technology and Education Center). Neither Ajo de Monte nor extracts or preparations of the plant have been tested for effects on cancer cells or other *in vitro* assays. Additionally, although no documented sources confirm Ajo de Monte’s effects on cancer, Davila et al. (2008) reported that Ajo de Monte contains several organosulfur ingredients that also occur in garlic, these organosulfur compounds are correlated with the lower

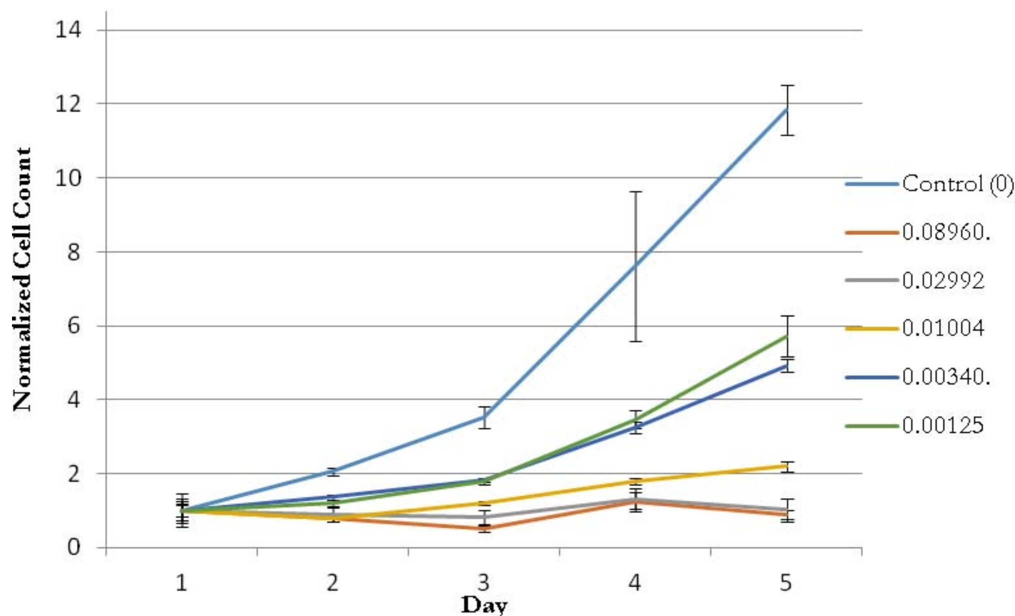

Figure 3. NIH Swiss Growth. Mean normalized cell count of NIH Swiss non-cancerous mouse cells each day in response to various levels of *M. alliacea* treatment (0 to 0.0896 g/ml). Normalized cell count produced by dividing the quantity of cells on a given day by the initial quantity of cells. “Control (0)” implies that water was added to the medium with no extract.

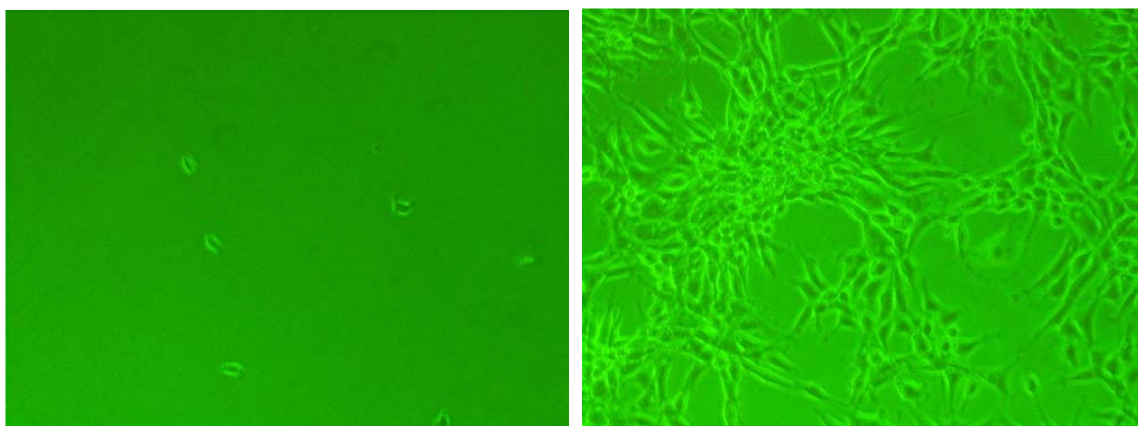

Figure 4. T3-HA Treatment Comparison. A comparison of quantity of cultured T3-HA cells from Experiment 2 on day five, with cells receiving the highest treatment level on the left, and those receiving the lowest treatment on the right.

incidence of cancers in clinical trials (Dorant et al., 1993; Thomson and Ali, 2003; Sakamoto et al., 1997; Wang et al., 2010). Of these, the chemically potent diallyl sulfides comprise 65.9% of the volatile compounds of Ajo de Monte and diallyl trisulfides 29.6% (Granados-Echegoyen et al., 2014). Additionally, these sulfonic compounds in garlic inhibit hepatic cholesterol synthesis, resulting in lower levels of systemic cholesterol with garlic consumption (Yeh and Liu, 2001). The reported success of these sulfonic compounds in garlic suggests that since both Ajo de Monte and garlic possess organosulfur compounds, it is possible that these plants share a common mechanism of action.

Likewise, the chemical compound allicin in Ajo de

Table 1. Concentrations of extract added to each of five treatment plates with one control.

Plate #	Media and water -900 µl H_2O -10 ml cell culture media	Extract and media -900 µl Ajo de Monte extract -10 ml media	Extract concentration (g dry weight leaf powder/ml)
1	5	0	0
2	0	5	0.08960
3	3.33	1.67	0.02992
4	4.44	0.56	0.01004
5	4.81	0.19	0.00340
6	4.93	0.07	0.00125

Table 2. ANOVA (analysis of variance) indicating statistically significant (<.001) difference between control and treatment cell populations.

Parameter	Sum of squares	df	Mean Square	F	Sig
Between groups	4788.498	5	957.700	11.175	0.000
Within groups	8741.068	102	85.697	-	-
Total	13529.567	107	-	-	-

Monte has been proposed to explain its anti-microbial properties. Song and Milner (2001) reported that alliin and the enzyme alliinase combine to produce allicin, a plant-defense chemical with a short half-life which they suggested to be responsible for the anti-microbial and anti-cancer properties in garlic. According to Davila et al. (2008), Ajo de Monte has higher concentrations of alliin in the roots and leaves than garlic. Since allicin also occurs in large quantities in Ajo de Monte, it has been associated with the anti-microbial properties of this species.

Allicin and the related allyl sulfide or diallyl sulfide constituents of Ajo de Monte and garlic have been identified with tumor suppression (Song and Milner, 2001; Zhou and Mirvish, 2005). Although the particular mechanism of action has not been completely defined, studies suggest that allyl sulfides prevent carcinogenesis as well as promote apoptosis of cancer cells. Allyl sulfides can prevent the attachment of the metabolite 7,12-dimethylbenzene(a)anthracene (DMBA) to rat mammary epithelial cell DNA (Song and Milner, 2001). Additionally, Zhou and Mirvish (2005) implied that allyl sulfide compounds prevented guanine methylation by nitrosamine compounds in rats, resulting in fewer neoplastic-generating base mismatches. Moreover, once cancer cells have developed, allyl sulfides can arrest cells in G_2 – M phase through Cdc25C signaling (Xiao et al., 2005). Knowles and Milner (2000) suggest that these compounds may play a role in increasing cellular membrane fluidity thereby suppressing integrin glycoprotein IIb-IIIa mediated adhesion, ultimately leading to cell cycle arrest and leading to premature apoptosis (Seki et al., 2012). Alternatively, allyl sulfides and polysulfides can be biologically cleaved to produce reactive oxygen species that may result in an anti-proliferative effect for cancerous cells by promoting apoptosis if they are sufficiently concentrated (Filomeni et al., 2008). Xiao et al. (2003) found that allyl sulfides decrease tubulin polymerization in cancer cells, ultimately leading to the structural collapse of the cell. Furthermore, Yeh and Liu (2001) reported that these organosulfur compounds are water soluble.

Allicin or a related polar organosulfur compound may be the active ingredient for these neoplastic effects. Since extracts of these compounds from similar species are reported to be successful in cancer treatment as well as other ethnobotanical uses, similar effects may also occur from extracts of Ajo de Monte.

Based on these results, Ajo de Monte extract has been shown to both inhibit the growth rate of T3-HA cancer cells at low concentrations and to kill colonies of cancer cells at higher concentrations. Therefore, Ajo de Monte could be used in the treatment of certain cancers due to its targeted killing of cancer cells. Besides killing cancer cells, the analgesic effects of garlic vine could prove to be beneficial for cancer patients. Moreover, the fungicidal and antibacterial properties of the plant may be used for the treatment of secondary infections. Further *in vivo* studies would be required to determine the effectiveness of Ajo de Monte in targeting cancer cells and the appro-

priate dosage required. Due to the high concentration of allicin in Ajo de Monte and other phytochemicals in this species, additional studies should be considered to determine their effectiveness in cancer treatment.

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Conflict of interest

Authors have none to declare.

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