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Effect of Snow Mountain Garlic Extracts on Cellular Count and Viability in Cell Lines Cervical Cancer

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ABSTRACT:

Introduction: Some varieties of garlic - such as the Snow Mountain variety - have been attributed a greater amount of medicinal effects than the rest of the *Allium* genus, due mainly to the amount of sulfur compounds they contain. The amount of these compounds depends - among other circumstances - on the extraction conditions and techniques.

Objective: To assess the effects of Snow Mountain garlic extracts, obtained by different processes, on count and cell viability in Cervicouterine Cancer cell lines.

Material and methods: Three extraction methods were used to obtain sulfur compounds; three different concentrations of each extract were prepared and their effects were evaluated on two neoplastic and one non-neoplastic cell lines. The evaluation methods were Neubauer chamber cell count at 24, 48 and 72 hours and a MTT test at 72 hours.

Results: A significant dose-dependent increase in the number and viability of neoplastic cells was observed ($p = 0.0001$ and $p = 0.0003$ respectively), specially in soxhlet extraction treatments.

Conclusion: the ethanolic, chloroformic and aqueous Snow Mountain garlic extractions show an increase in the number and viability of Cervicouterine Cancer (CuCa) cells, contrary to other *Allium* varieties, so their probable anti-neoplastic activity requires further study.

Keywords: cancer, *Allium sativum L.*, Snow Mountain garlic, cell count and viability.

1. Introduction

Snow Mountain garlic, also known as Japanese garlic or Himalayan garlic (Zahoor, 2018), is a very particular variety of the *Allium* genus that has often been identified as *Allium sativum* or *AlliumsativumL.*, a term that has also been used to refer to other varieties such as purple creole garlic (Mujica, Pérez, Sanabria and Giménez 2013), garlic obtained in mediterranean markets (Bocchini, Andalo, Ppzzi, Glleti and Antonelli, 2001), various varieties of Iranian garlic (Baghalian, Ali, Reza, Naghdi and Khalighi, 2005), the Chinese "De Zhou Hong Pi" garlic variety (Wang *et al.*, 2014), among other varieties, and that have been used in studies oriented to the determination of therapeutic properties of the species.

What distinguishes Japanese garlic from the rest is that it only has one bulb (chive-shaped) of approximately 2 to 5 mm (Fig. 1), and not multiple bulbs as in most *Allium* varieties. The Snow Mountain bulb is surrounded by a thin and transparent (a) inner membrane that covers and protects it as in the rest of the species; it also has a (b) stiffer dark brown middle layer and a (c) rigid outer layer simulating a rigid shell. This outer layer is light yellow at early stages, but as it matures it becomes dark brown.

This external cover favors the survival of the species, since it grows in tremendously hostile environments: -10 ° C temperatures, very little oxygen and high pressures - 60,000 feet above sea level- (Mahajan, Sharma, Bandrval, Jamwal and Billowria, 2013). The ability of Snow Mountain garlic to survive in unfavorable environments has given it many medicinal properties and health benefits, even above other garlic species. For example, in past times mountaineers used it to improve their energy levels and detoxify their body in extreme cold (Zahoor, 2018).

Currently, various websites describe the beneficial properties of Japanese garlic to human health, including; anticancer activity, cholesterol reduction and protection of liver functions. However, there are no studies reported with this variety of garlic that support evidence of these benefits.

What is well identified is a wide range of chemical substances found in all *Allium* species with biological activity, such as organosulfides, to which the therapeutic properties of the species are attributed. Santhosha, Jamuna and Prabhavathi (2013) point out, for example, that garlic - without determining the variety - contains approximately 65% water, 28% carbohydrates, 2.3% organosulfides, 2% protein, 1.2% free amino acids and 5% fiber. Other authors (Lutomski, 1987; Lawson, Wood and Hughes, 1991; Lawson, Wang and Hughes, 1991; Kamenetsky*et al*., 2005; Méndez and Castaigne, 2008; Soto, González, Sance, Burba and Camargo, 2010; Hassan, Haiping, Xinya and Xixiang, 2015) mention that these compounds can vary in fresh garlic from one species to another, from the time of the year of the harvest, the extraction conditions (temperature, time and polarity of the solvents) and storage conditions (Bose, Laha and Banerjee, 2014; Chhouk, Uemori, Wahyudiono, Kanda, and Goto, 2017).

The main organosulfide of intact garlic is alliin (S-allylcysteinesulfoxide), an alkali derivative of alkali-cysteine sulfoxide in concentrations of 0.2 to 2.0%. Together with other sulfoxides, alliin is part of the plant's defense mechanisms against pathogenic microorganisms (Borlinghaus, Albrecht, Gruhke, Nwachukwu and Slusarenko, 2014); In the presence of damage to cell membranes, alliin is hydrolyzed by the enzyme alliinase, producing allicin.

Allicin represents 70 to 80% of the total thiosulphinates formed by allinase. It has an oily consistency and a light yellow color (Ilic*et al*., 2012) and is the cause of the unpleasant smell and spicy taste of garlic, and the molecule to which its antibiotic, antifungal, pesticide and anticancer properties are attributed (Singh and Singh, 2008). Slusarenko, Patel and Portz (2008) point out that a single bulb of garlic of approximately 10g produces about 5 mg of allicin and, according to Wang, Li, Liu and Jin (2009), in order to obtain a therapeutic effect, 4.5mg g^{-1} of allicin is required to be present in an extract.

The extraction method then, has an important impact on the biological activity, and the amount of sulfur compounds obtained from garlic (Singh *et al*., 2008), particularly allicin since it is a highly volatile and unstable compound, which degrades rapidly into more stable molecules depending on the solvents and techniques used, as well as a variety of conditions.

Among the main solvents used for the extraction of organosulfides are methanol and ethanol (Cavallito and Bailey, 1944; Block, 1992; Yu, Wu, and Ho, 1993;Artacho, Olea and Ruiz, 1995),that favor the presence of volatile compounds in the extract. Due to the non-polar characteristics of allicin, it has also been obtained using the soxhlet method, as well as aqueous extractions from garlic macerations (Wang *et al*., 2015).

Objective:

To evaluate the effects of Snow Mountain garlic extracts, obtained under different conditions, on cell count and viability in cervical cancer cell lines.

2. Materials and Methods

2.1 Cell lines

SiHa, HeLa and HaCaT (non-neoplastic cells) cell lines grew in RPMI-1640 medium supplemented with 10% fetal bovine serum (FSB) + 1% penicillin (10,000 units/ml)/streptomycin (10,000 µg/ml), and kept in an incubator at 37 °C in an atmosphere of 5% CO_2 and controlled humidity.

2.2 Garlic Extracts

Fresh bulbs of Snow Mountain garlic were obtained from the local market and stored at room temperature until processing.

2.2.1 Southern Cross University Phytochemical Analysis Laboratory (SCUPAL) Method: 100 grams of fresh garlic were dried over two weeks at 40 °C in a drying oven and then pulverized in a mortar. 6 grams of garlic powder were mixed with 30 ml of absolute ethanol in a vortex for 20 seconds and the mixture was subjected to sonication in a DIAGGER apparatus (Branson). The samples were subjected to two cycles of 10 minutes, each at 22 °C and then centrifuged at 5,000 rpm for 30 min. The liquid was decanted and the vessel covered with a porous membrane for evaporation of the solvent at room temperature (Díaz and Jiménez, 2008; Chhouk*et al*., 2017).

***2.2.2 Soxhlet method*:** 80g of peeled garlic, finely cut and wrapped in filter paper were placed as a porous cartridge in the soxhlet siphon; 700 ml of chloroform were used for extraction for 8 hrs in a Barnstead/Electrothermal BI extractor. The solvent was evaporated at room temperature for 24 hours, and the container was protected from light and kept refrigerated at 8 °C until use.

***2.2.3 Aqueous extraction*:** The last preparation consisted of 2.75g of fresh garlic crushed in a mortar at the time of the test; 3ml of distilled H_2O was added to the crushed garlic, the mixture was homogenized with a pipette and filtered on a MILLIPORE microfilter.

2.3 Cytotoxicity test

The treatments were prepared in three concentrations of each extract by diluting the product in 0.01% DMSO. The products were named according to the solvent used for the extraction. The concentrations were as follows:

	C1	*C2*	*C3*
Ethanol	31µg/µl	62µg/µl	93 µg/µl
Chloroform	75µg/µl	150µg/µl	225µg/µl
Water	45µg/µl	90µg/µl	135µg/µl

$1x10^4$ cells of two CuCa cell lines (SiHa and HeLa) and one non-neoplastic line (HaCaT) were seeded in a 24-well microplate, there were two control wells (positive and negative) and the three concentrations of each extract for each line. All trials were completed in duplicate.

For the cell count, a trypan blue exclusion test and a Neubauer chamber count were performed at 24, 48 and 72 hours. Viability was assessed by the MTT (Thiazolyl Blue Tetrazolium Bromide 5mg/ml) test at 72 hrs on a microplate spectrophotometer, with an excitation range of 570 nm and an emission range of 596 nm.

The data obtained were processed in the GraphPad Prism version 7 software; a Kruskal-Wallis test was used for statistical analysis.

3. Results

3.1 Cell count

When analyzing the effect of Snow Mountain garlic by extract, on the cell count of each line (Fig. 1), we observed that the SiHa line (A) showed the greatest number of cells, mainly in those treated with the chloroform extraction, but without statistical significance. In the (B) HeLa line, meanwhile, lower figures

than SiHa were observed but the chloroform extraction maintained the highest number, in contrast the HaCaT (C) line recorded the lowest figures.

In the statistical analysis of the effect of Snow Mountain garlic as a function of concentration in neoplastic and non-neoplastic lines (Fig. 2), we found that SiHa cells showed a significant increase in C_3 ($p = 0.0003$) in relation to the control, while in HeLa cells there was a significant decrease in C_1 ($p = 0.0001$), the HaCaT cells, meanwhile, remained low in number.

3.2 Cellular viability

In Fig. 3 (A), greater viability is observed in CuCa cells treated with all allicin extracts, while HaCaT cells expressed low viability. However, HeLa cells showed a significant decrease in viability in C_1 ($p = 0.04$), which is consistent with what was found in the cell count. On the other hand (B), a greater viability of CuCa cells treated with aqueous extract was observed and, in accordance with the cell count, the HaCaT line maintained low viability in all treatments and with all extracts.

4. Discussion

The results obtained in the present study differ from those reported in the work of Zhang and Yang (2019) in which they show that treatments with 5, 20 and 50 nm of allicin suppress the viability of SiHa cells in a dose and time dependent manner. However, the authors do not report whether the allicin used was purified or was present in extracts for the treatments, nor which preparation methods were performed. They also don't discuss the effects of these concentrations on non-cancer cells. Other studies that report similar results have been carried out on other types of cancer cell lines: colon (Lee *et al*., 2013), promyelocytic and myelomonocytic cells (Miron*et al*., 2008), lung, breast and colonor rectal cancer (Gruhlke, Nicco, Batteux and Slusarenko, 2017).

On the contrary, Li *et al*. (2015), found an increase of chondrocyte proliferation when treated with garlic extract (they do not mention the method) in concentrations of 10 to 50 µg/ml and measured by an MTT test at 12, 24, 36 and 48 h. The authors report, even an increase in S phase cells at 36 h in the 40 µg/ml treatments. Similarly, Chen, Pang, Lin, Xia and Wang (2016), report a significant increase in the viability of umbilical vein endothelial cells (HUVEC) pre-treated with 10, 30 and 100 µM allicin (they also don't discuss the extraction method), and exposed to oxidized low density lipoproteins (ox-LDL) - which induce endothelial damage -. They show that allicin had a protective effect on HUVEC cells due to the reduction of apoptosis induced by endothelial damage, and they indicate that this effect is related to the inhibition of caspase-3 and to the apoptotic signaling related to NADPH oxidase.

Other similar studies (Sundaram and Milner, 1996; Mehri, Auger and Bauvois, 2008; Ruiz-Cabello, Puerto, Gutiérrez-Praena, Pichardo and Cameán, 2013) report having found no decrease in cell viability in cancer lines treated with extracts of garlic and/or allicin. The studies reviewed lack specificity with respect to the variety of *Allium* used in extracts, and therefore the concentrations of allicin or organosulfides tested.

5. Conclusions

The three extractions (ethanolic, chloroformic and aqueous) of Snow Mountain garlic tested in the present work increased the number and viability of CuCa cells, particularly in concentrations above 31 µg/µl, this was not observed in non-neoplastic cells (HaCaT). No significant differences in these effects were observed between the extracts used.

Snow Mountain garlic seems to have a restrictive action both in the number and in the viability of non-neoplastic cells, which may indicate a protective effect against cancer, however, more studies are required to prove it.

Figures

Figure 1. Snow Mountain Garlic.On the left, the rounded shapes of the bulbs are observed with a flattened face that ends at a peak, while bulbs of different sizes are found; on the right, garlic protections are shown: (a) the inner membrane is a thin transparent layer that allows to see the whitish slice (b) the middle layer consists of a semi-rigid brownish brown structure that is attached to the inner membrane, (c) the outer layer of solid consistency that varies from yellow to dark brown depending on the maturation of the bulb.

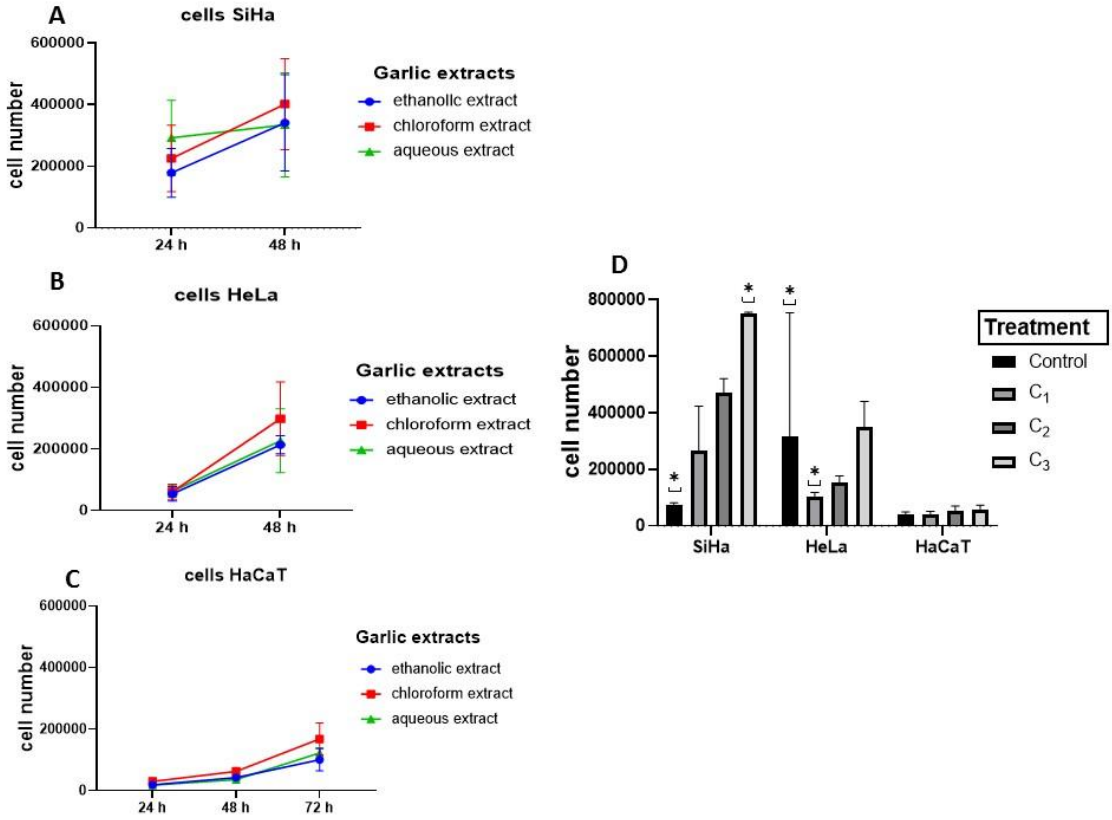

Figure 2.Neoplastic and non-neoplastic cell count treated with Snow mountain garlic extracts. An increase in the number of neoplastic cells - SiHa (A) and HeLa (B) - was observed from 24 to 48 h at all concentrations, mainly with the extract in which chloroform was used as a solvent, while ethanolic extracts and Aqueous behaved similarly. The number of non-neoplastic cells - HaCaT (C) - also shows an increase in all concentrations with the three extracts; however, note that the increase in this line was observed until 72 h, although well below the figures found in the neoplastic cells; there were no significant differences between the increase found in the three extracts in any of the cell lines. (D) Figure 2D comparison of the number of cells treated with Snow Mountain garlic extracts per cell line. SiHa cells show, in general, a significant increase ($p = 0.0003$) dose-dependent in C3 treatments of all extracts with respect to the control; while in HeLa cells a significantly lower number ($p = 0.0001$) was found in C1. HaCaT cells, treated with the different concentrations of extracts, show a control-like growth without statistical differences ($p > 0.9999$).

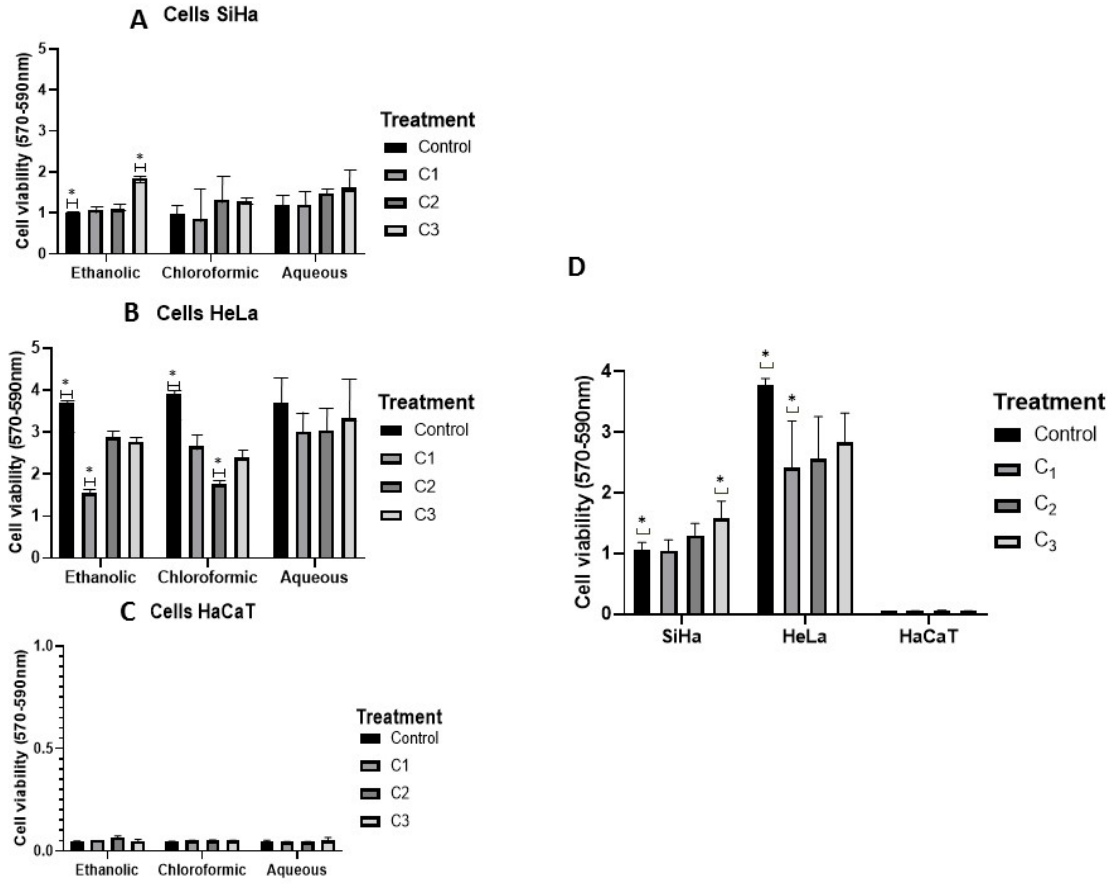

Figure 3.Viability of neoplastic and non-neoplastic cells treated with Snow Mountain garlic extracts at 72h. The MTT test results show an increase in metabolically active SiHa (A) cells in the C3 treatments of all extracts, but only reached statistical significance (p = 0.0003) in the ethanolic extract. In contrast, in HeLa (B) cells, lower viability is observed in C1 of the ethanol extract (p = 0.04) and C2 of the extract with chloroform with respect to the control. For HaCaT (C) cells the viability in the treatments remained very similar to the control, without statistical differences (p> 0.5). When comparing the viability of the treated cells with all extracts by cell line (D), we also found that SiHa showed a greater (p = 0.0003) number of viable cells in C3 compared to the control, while HeLa exhibited less viability in C2 and the treated HaCaT cells behaved similar to the control without statistical differences (p> 0.5) between them.

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