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Anti - tumorigenic assessment of HELA cell growth characteristics in the presence of Meswak

Azhaar N Anber Altayyr
Clark Atlanta University

ANTI-TUMORIGENIC ASSESSMENT OF HELA CELL GROWTH CHARACTERISTICS IN THE PRESENCE OF MESWAK

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AZHAAR N. ANBER ALTAYYR

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ABSTRACT

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ANBER ALTAYYR, AZHAAR N. B.S. TAIBAH UNIVERSITY, 2011

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Committee Chair: David Logan, Ph.D.

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There is a demand for newer, more effective therapeutic antagonist for bacterial threats. More specifically, overuse of currently available antibiotics, premature dosage-termination of prescription antibiotics and a growing resistance of bacteria to currently available antibiotics warrants the design, development and establishment of new and novel antibacterial therapeutics. Unfortunately, physician–scientists no longer have an antibiotic agent for an increasing number of multiple strains of bacteria. In this study, we investigate the holistic efficacy for which the plant Meswak can antagonize the vitality of some strains of bacteria and HeLa cell growth. The conclusions drawn from the findings of this investigation suggest Meswak as a potential antagonist for the growth of some strains of gram-positive and gram-negative bacteria. Preliminary studies have also revealed that Meswak acts as an inhibitor to HeLa cell growth and migration. Future studies are necessary to define the specific mechanism of action for which Meswak acts to inhibit bacterial viability and HeLa cell growth and migration.

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CHAPTER I

INTRODUCTION

Growing issues and concerns of antibacterial drug resistance necessitates a need for contemporary and novel antimicrobial therapeutics (Al-Samh and Al-Nazhan, 1997). Rising resistance to currently available antibiotics has spurred the onset of new and untreatable strains of bacterial diseases (Al-Samh and Al-Nazhan, 1997). The establishment of better and more effective antimicrobial therapeutics is predicated upon the ideas that stem from well-funded research for support of pursuit of modern countermeasures to current drug-resistance issues. Perpetuating this effect has been the world-wide overuse of antibiotics causing the spread of bacterial resistance. Wide resistance to antibiotics prompts the necessity for newer, effective antibiotics. In this study, we seek to investigate the antimicrobial properties of Meswak on HeLa cells in vitro as a potential antagonist for bacterial growth over time courses of 24 hours.

Native to the Middle East, Meswak (Salvadora *persica)* is a Peelu tree twig popularized for its medicinal benefits for teeth cleaning and maintenance of good oral hygiene. Predominant in Muslim-inhabited areas, Meswak heralded fame and recommendations of use by the prophet Muhammad. Although the scientific community lacks a body of compelling research findings that which either discredit or support the claims of hygienic and medicinal properties of Meswak, the implications of its efficacy and potency are remarkable. In a 2014 publication regarding the medicinal properties of

Meswak on common oral pathogens, findings of antimicrobial properties were reported as exhibited by Meswak on more than 100 health care workers (Naseem et al., 2014). Another publication reporting on the antibacterial potential of Meswak was observed in the presence of two strains of bacteria without eliciting effects of toxicity (Al-Sieni, 2013). Meswak was also reported to exhibit antibacterial inhibitory effects on both gram-positive and gram-negative strains of common oral bacteria (Sukkarwalla et al., 2013). Benzyl isothiocyanate was identified as the active component of Meswak responsible for antagonistic effects on gram-negative bacteria (Sofrata et al., 2011).

The isothiocyanate isolated from Meswak exhibited selective inhibition of mutant p53 in breast, colon and prostate cancer cells *in vitro* (Wang et al., 2011). This compound functioned to introduce apoptosis in diseased cells but not normal cells as identified by modified p53 (Wang et al., 2011). Further elucidation of the functional role of isothiocyanates resulted in cell cycle arrest and cessation of cell growth (Mi et al., 2008). More specifically, microtubule assembly in human lung cancer cells was inhibited *in vitro* and *in vivo* by isothiocyanates (Mi et al., 2008). Collectively, the isothiocyanates act on cancer cells to inhibit multiple hallmarks of cancer.

CHAPTER II

MATERIALS AND METHODS

Meswak Preparation

Unprocessed Meswak was purchased and obtained in the Kingdom of Saudi Arabia and imported to the United States during international passenger travel. In preparation for shipment, twigs were separated from the total plant, sundried and stored in a cool and dry satchel of camel hide. In the Environmental Biology laboratory at Clark Atlanta University in Atlanta, GA, the brown fibrous and pliable Meswak twigs were cut into small 1 cm segments before they were ground into a fine powder in a ceramic mortar and pestle. Calculations for dilutions were performed to determine the mass of Meswak necessary to achieve an isotonic concentration that would be administered *in vitro* to HeLa cells.

For the preparation and reconstitution of experimental Meswak stocks done in-house, 0.9g of finely ground Meswak was resuspended into 100mL of ddH_20 for trial one of water-based extract and again into 100mL of 95% denatured ethanol obtained from EMD Millipore for trial one of alcohol-based extract. Both reconstitutions were established in glass beakers containing separate metal bars and situated atop of stir plates. After achievement of miscible reconstitutions, solutions were permitted to stir under low heat for 30 minutes for the homogenous dispersal and dissolution of Meswak. Extracts were autoclaved at 121°C for 15 minutes then cooled to room temperature. Samples were

separately syringe-filter sterilized using a 0.2μM filter. Stock solutions were stored between 2°C and 8°C in a dark refrigerator. Dry powder extract was stored at room temperature in a sealed polyethylene conical tube.

HeLa Cells

HeLa cells were purchased from American Type Culture Company as HeLa (ATCC CCL-2). The base medium that accompanied these cells was ATCC-formulated Eagle's Minimum Essential Medium supplemented with 10% heat –shocked fetal bovine serum. Medium and fetal bovine serum was separately syringe-filter sterilized with 0.4μM and 0.2μM filters respectively before combining.

Cells were revived from cryopreservation of 5% (v/v) DMSO supplemented with complete growth medium. Cells were cultured in-house at Clark Atlanta University in the Molecular Biology C.O.R.E. for Cell Culture. Using Corning CellGro T-75 cm^2 flasks, revived cells were thoroughly resuspended and seeded at densities of 2.0×10^6 cells per flask. Flasks of HeLa cells were incubated at 37°C in 5% CO_2. Medium was renewed between two and three times each week according to presence of debris in supernatant or color change that indicated drops in pH. Cells were passaged for two passages in order to establish pre-requisite populations of cell densities necessary for experimentation.

In some instances, HeLa cell culture medium was supplemented with 100 μg/mL of penicillin/streptomycin for maintenance of healthy cells. Supplements of penicillin/streptomycin were suspended 24 hours prior to the start of and conduction of each experimental study as not to positively or negatively impact the results observed in studies Scratch assay.

HeLa cells were cultured to 70% - 80% confluency for the establishment of stabilized cells and harvested near the apex of the growth phase. Cells were plated at a density of 3.85×10^5 cells per dish 24 hours prior to the start of experimentation for the establishment of a uniformed monolayer of cells under aseptic conditions. All residual media was removed from the culture flask. Cells were rinsed once with complete media or phosphate buffered saline to ensure the removal of dead or loose cells and debris.

Plates were inverted as single and straight lines were sketched down the equator of each plate. Plates were returned to right-side up before using separate sterile toothpicks for each plate to scratch-trace single and straight lines down the equator of each plate consistent with the line drawn on the underside that displaces the monolayer of cells only along the equatorial line. Cells were washed with incomplete media or phosphate buffered saline twice to remove dead or loose cells. Cells were supplied complete media prior to receiving vehicles or treatments with Meswak extracts.

HeLa cell status of migration was photographed at different time points of 3, 6, 12 and 24 hours in the presence of Meswak in comparison to vehicle controls. Cells were observed and imaged using an Olympus CHK Compound Microscope at 20X magnification. Immediately following each time point, cells were harvested from culture plates using 0.25% Trypsin-EDTA, centrifuged, resuspended and counted for the quantification of cell viability and necrosis using the Nexcelom Cellometer cell counting machine and slides.

Viability

HeLa cells were cultured to 70% - 80% confluency for the establishment of stabilized cells and harvested near the apex of the growth phase. Cells were plated at a density of 1.08×10^6 cells per dish 24 hours prior to the start of experimentation for the establishment of a uniformed monolayer of cells under aseptic conditions. All residual media was removed from the culture flask. Cells were rinsed once with complete media or phosphate buffered saline to ensure the remove of dead or loose cells and debris.

HeLa cell status of viability was quantitated at different time points of 3, 6, 9, 12, and 24 hours in the presence of Meswak in comparison to isotonic controls. Immediately following each time point, cells were harvested from culture plates using 0.25% Trypsin-EDTA, centrifuged, resuspended and counted for the quantification of cell viability and necrosis using the Nexcelom Cellometer cell counting machine and slides.

CHAPTER III

RESULTS AND OBSERVATIONS

The reconstitution of Meswak into an isotonic solution was carried out by a passive solid – liquid extraction process. Meswak was obtained as a fibrous solid from roots and limbs of the tree from which Meswak is derived. A commercial-grade, kitchen blender was used to help generate a fine powder of Meswak. Benzyl isothiocyanate was extracted from the Meswak powder. Soluble in DMSO and ethanol, the benzyl isothiocyanate from Meswak hydrolyzes in water. Therefore, Meswak was carefully introduced into isotonic extracts of a water base and again of an ethanol base (denatured to 95%).

HeLa cell culture was maintained in CellGro T-25 treated dishes for tissue culture. Cell culture protocol was optimized in order to accommodate optimum growth of HeLa cells. Cells were passaged no greater than seven passages for each experiment. HeLa cells were sub-cultivated at a dilution of 1:2 – 1:6 according to the demands of each experiment. Media was renewed every two days. A cocktail of penicillin and streptomycin were added for the maintenance of HeLa cells in culture in order to combat and deter bacterial contaminations. Penicillin-streptomycin antibiotics were suspended after HeLa cells were plated for experimentation. Only cells reserved for culture remained in the presence of antibiotics during normal tissue culture.

Scratch Assay

HeLa cell cultures sustained minimal inhibition of growth in the presence of Meswak. After 24 hours of incubation, Meswak inhibited the growth of some HeLa cells at varying concentrations in comparison to the controls as shown in Figure 1. Meswak-ethanol extracts exhibited the largest inhibition of migration of HeLa cells over 72 hours in comparison to Meswak-water controls.

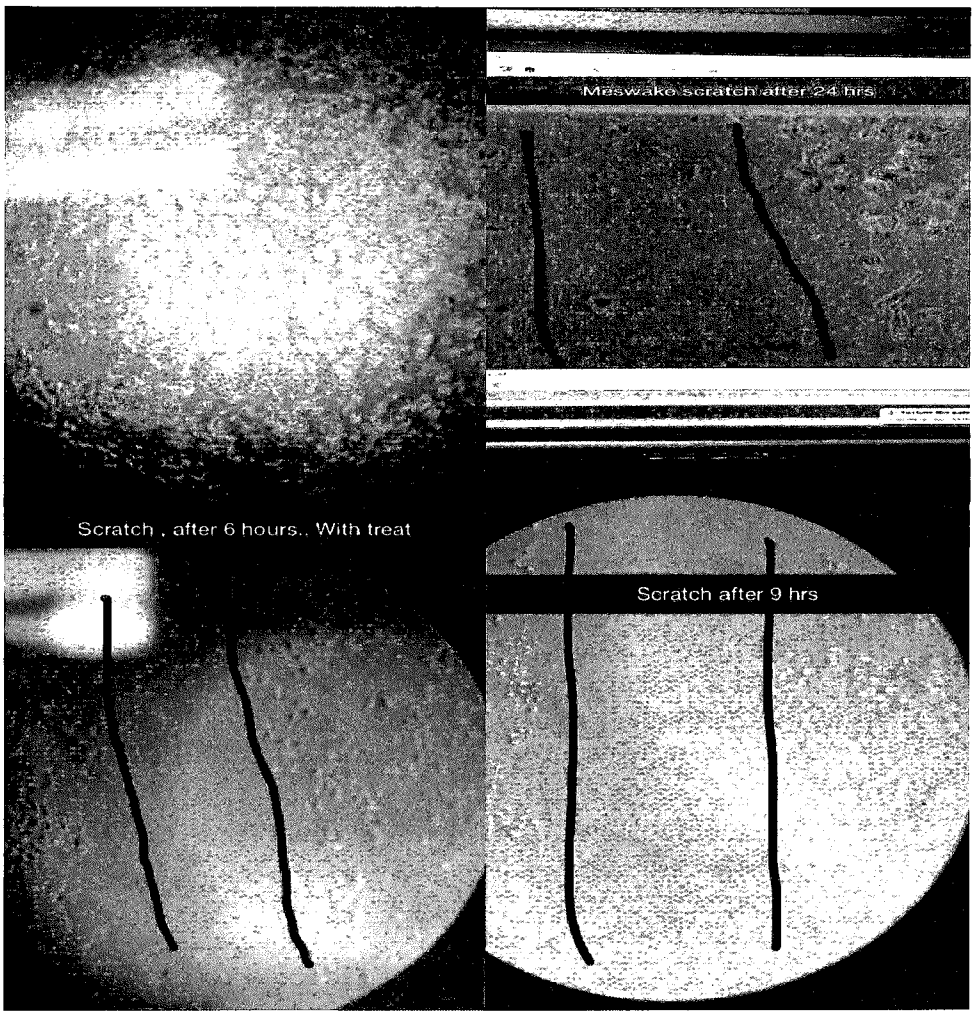

Figure 1: Migration results of HeLa cell scratch assay in the presence of Meswak extracts.

Cell Viability Assay

Scratch wound healing assays revealed low levels of migration of HeLa cells as early as three hours after scratching of the monolayer of HeLa cells in isotonic controls. After 9 hours, moderate levels of inhibition for migration was observed among HeLa cells treated with 0.9% NaCl-extracts of Meswak. After 9 hours, moderate levels of inhibition for migration was observed among HeLa cells treated with 0.9% NaCl-extracts of Meswak (see Figure 2).

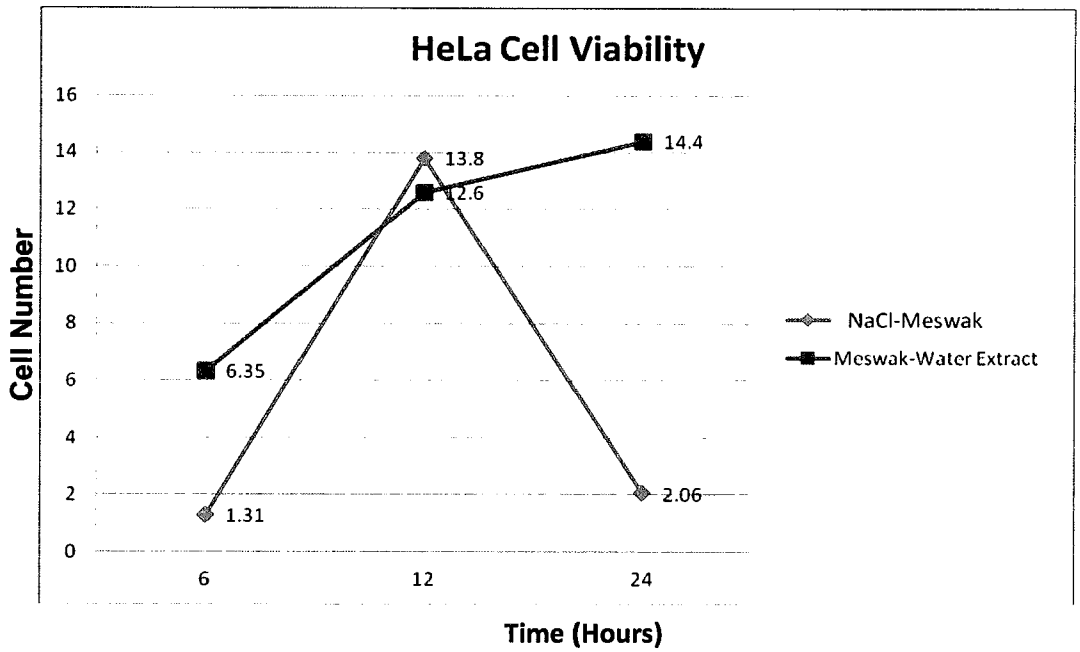

Figure 2: Comparison of HeLa cell viability after treatment with water and ethanol Meswak extracts over 24 hours.

Migration Assay

HeLa cells were seeded at density of 3.58×10^5 cells per dish. After a scratch, cells were permitted to grow for up to 24 hours. Dead cells were omitted from the cell

count by washing with phosphate buffered saline to remove them. An Olympus Compound Microscope was used to photograph cells for manual counting on a Dell computer.

Assessment of migration of HeLa cells in the presence of Meswak exhibited the greatest reduction in migration of HeLa cells after scratch as shown in Figure 3 as compared to the no treatment control. Sodium chloride vehicles demonstrated results similar to treatment with Meswak. HeLa cells treated with Meswak exhibited a reduced ability to migrate into the scratched area of culture dishes. When Meswak was not added to HeLa cells and the monolayer of cells scratched, HeLa cells quickly moved into the vacant area of the culture dishes.

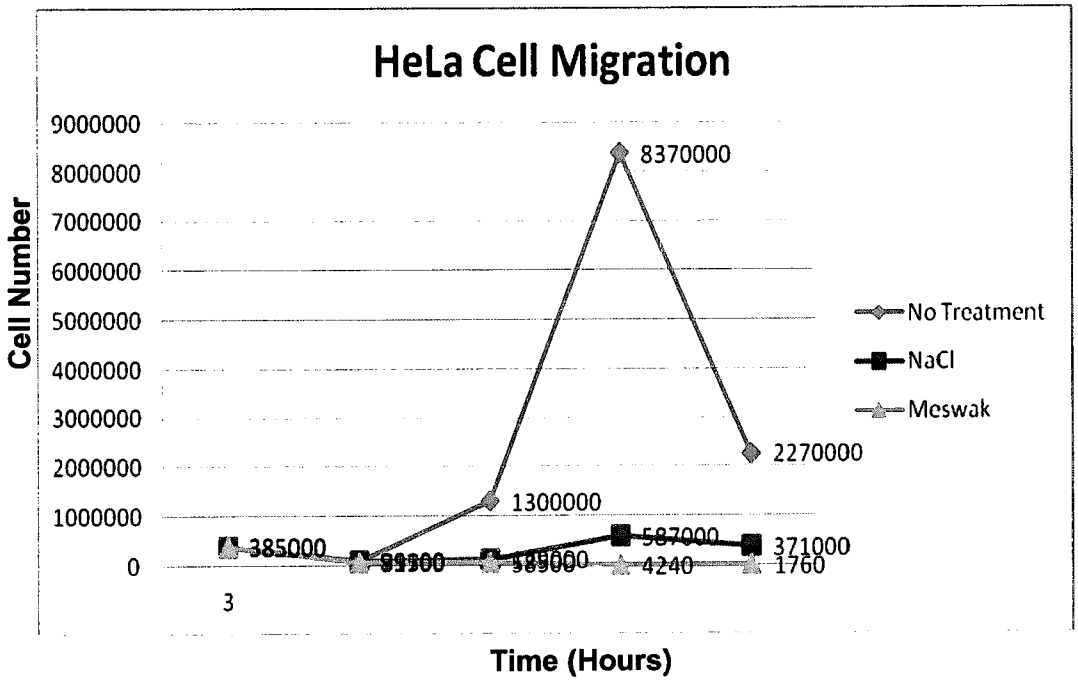

Figure 3: HeLa cell migration results.

CHAPTER IV

DISCUSSION

Reconstitution of Meswak into NaCl has been shown in our studies to be more effective or potent for inhibiting proliferation and migration of HeLa cells than reconstitution of Meswak into water alone. Furthermore, the isotonic solution of Meswak reconstituted into ethanol provided effects that were correlated to be more effective than the isotonic control of Meswak reconstituted with sodium chloride.

Observed results indicate that Meswak reconstituted into ethanol significantly reduced the ability of HeLa cells to migrate. This observed difference is related to the alcohol soluble constituents that were present. The water soluble extracts contained constituents that were not inhibitory to cell migration in HeLa cells. The monolayer of HeLa cells scratched experienced minimum migration of cells into the vacant space after 24 hours. Supplemental data not reported here resulted in HeLa cells also minimally migrating into the vacant areas of scratched monolayer of cells.

Observed results for HeLa cell viability in the presence of Meswak resulted in the least amount of viable cells. This finding suggests directly that Meswak has the potential to reduce the rate of proliferation of HeLa cells as early as three hours after treatment. This finding does not suggest that Meswak induces apoptosis in HeLa cells. Future studies should work to assess this question.

CHAPTER V

CONCLUSION

The preparation of an isotonic solution was necessary in order to assure cell viability in terms of osmotic pressure in the cells. In other words, an isotonic solution consistent with physiological pH and osmolality would function to deliver the Meswak compound without compromising the integrity of the pressure-volume gradient maintained about cell membranes. The Meswak solution concentration used for this study is physiologically stable and does not produce changes in terms of the cellular constituents and ~~of~~ typical physiological conditions for HeLa cells grown in culture.

Meswak is derived from the limbs and roots of the evergreen shrub, *Salvadora persica.* The fibrous and woody tissue contains the ingredients heralded to provide antibiotic and antimicrobial activities. These activities have been linked in other studies to the characteristic properties of isothiocyanates which are also present inside of Meswak (Sofrata et al., 2011). More specifically, benzyl isothiocyanate was identified as the primary active ingredient isolated from Meswak for its antibacterial properties (Sofrata et al., 2011).

A natural component of many cruciferous vegetables, isothiocyanate contains a benzene ring single bonded to a nitrogen atom that is double bonded to a carbon atom

that is double bonded to a sulfur atom (Khatak et al., 2010) as depicted in Figure 4. This compound is formed when the enzymes glucosinolates substitute sulfur atoms for oxygen atoms (Dains et al., 1926). Taken together, experimentation on for migration potential and viability in the presence of Meswak suggests Meswak to have a significant negative impact on HeLa cells. In the future, these studies should be carried out to include assessments of apoptosis, assessment of an inhibitory concentration and half-life of Meswak.

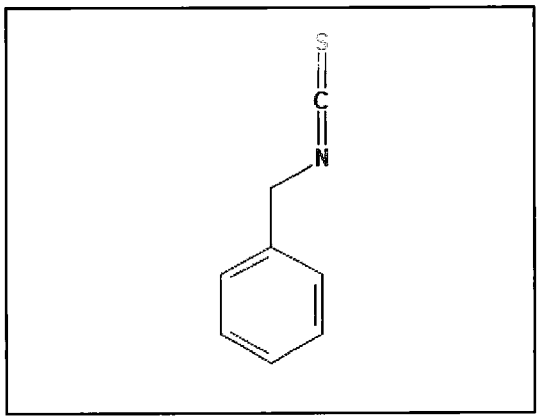

Figure 4: Chemical skeleton for isothiocyanate.

The extraction of isothiocyanate should be more involved than the process implemented in this study. The compound itself is hydrophilic in nature. This should also be taken into account during dehydration and extraction. While investigators of this study attempted to perform a solid-liquid extraction, the extraction process can be improved upon to produce a greater yield of extract as well as an extract that is more pure. Reconstitution of Meswak into water versus 95% denatured ethanol appears to be relatively more effective in treatments for inhibition of growth and migration of Meswak extracts performed similarly.

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