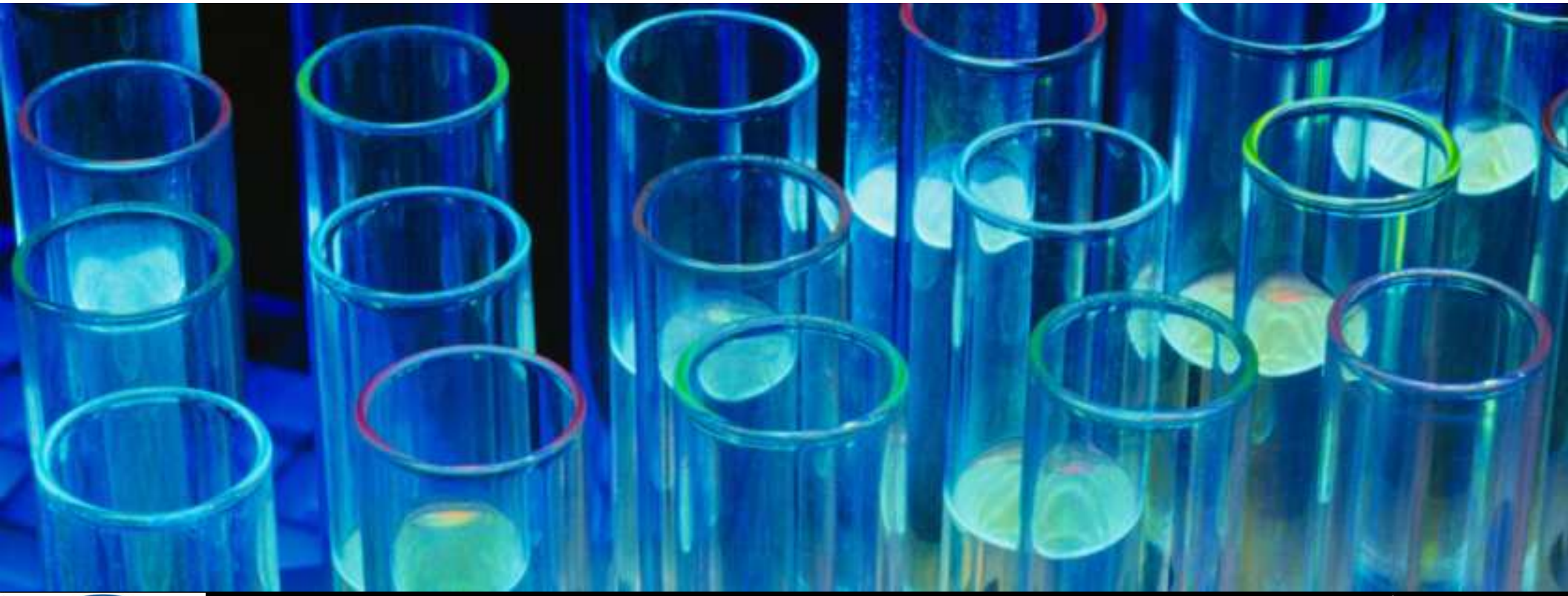

The Potential Intrinsic Anticancer Characteristics of Hops

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Project Overview

- Terpenes, α-acids, and β-acids derived from hops (*Humulus lupulus*) contribute to the flavour, aroma, bitterness, and preservative characteristics of craft beer. These molecules dissolve during the boil of the wort; however, the α-acids isomerize while terpenes and β-acids remain unchanged in the solution as it passes on to the final product. Some of these molecules, such as the terpene alpha-humulene, have been shown to inhibit the growth of cancer cells. This study investigates the contribution of these intrinsic bioactive components from hops in the potential anticancer activity of beer.

Materials

About the Beer used in this Study:

- *During the brewing of the wort, the oils (α-acids) of the hops become soluble as they isomerize while adding a bitterness characteristic to the beer. This bitterness can be can be quantitatively defined a International Bitterness Unit (IBU) which is equivalent to 1 mg/L or 1 ppm of iso-alpha acids.*

- **El Cerrito** is a Mexican style Lager made via *Saaz* hops with a 22 IBU index and a 4.75% ABV
- **Hollow Body** is a Indian Pale Ale made via a 50:50 ratio of *El Dorado* and *Citra hops* with a 40 IBU index and a 5.75% ABV.

The Hops used in this Study:

- **El Dorado** hops, grown in the Yakima Valley, “Washington Wine Country” and have a α-acid composition of 13-17% and a β-acid composition of 7-8%.

- **Citra hops**, also grown in the Yakima Valley have an α-acid composition of 11-13% and a β-acid composition of 3-4.5%.

- **Saaz hops**, grown out of the Czech Republic have an α-acid composition of 2.5-4.5% and a β-acid composition of 4-6%.

About the Cell Lines

- **HT-29** is a cancerous cell line established in 1964 from a 44 year old Caucasian female diagnosed with colorectal adenocarcinoma (epithelial colon cancer). This cell line is a benchmark for *in-vitro* studies. [1]

- **SCC-25** is a cancerous cell line from a 70-year-old adult male diagnosed with Squamous Cell Carcinoma (epithelial tongue cancer). This type of cutaneous malignancy is common and accounts for 20-30% of cancers associated with Caucasians. [2]

Methodology

Now Bear with Me…

Preparation of the Hops Solution

- To maintain the ratio of the amount of hops used during the wort (1 pound per 31 gallons) 0.760 grams of Saaz (El Cerrito) hops were boiled in 200 ml of filtered water for 30 minutes. Additionally 0.380 grams of Citra and El Dorado (Hollow body) hops were boiled in 200mL for 30 minutes. The solution was filtered via decanting after spinning at 4000 rpms for twenty minutes.
- The solution underwent sublimation to remove the moisture. (Freeze Drying)

Beer Treatment

- The beer was prepared by transforming it into a non-alcoholic beer by using heat and agitation to evaporate the ethanol from the beer while ensuring that a boiling temperature is not reached. The agitation helped to de-gas the carbon dioxide, but to ensure a full de-gas– the solution was subjected to a vacuum.
- The solution underwent sublimation to remove the moisture (Freeze Drying)

The Extract Variables

- Two beer samples (Hollow Body & El Cerrito) and two solutions containing only the hops (Citra/El-Dorado & Saaz) that were used to make the beer before being freeze-dried and resuspended in cell culture media at original brew strength and varying diluted concentrations.

Culturing the Cells

- HT-29 and SSC-25 cell lines were preserved in *McCoy's 5A* and DMEM:F-12 media ("USDA Certified, sterile"~Global Cell Solutions, Inc.), respectively. All media was enriched by an antibiotic solution diluted to 100 units/mL of penicillin and 100 µg/mL of streptomycin (Sigma-Aldrich Corporation) and a 10% fetal bovine serum (Atlanta Biologicals, Inc.). The cell line was sub-cultured by trypsinization to maintain growth in the log phase while under atmospheric conditions of 37°C at 95% humidity in a 5% CO2 incubation cabinet

Treating the Cells

The Cancer Cells were incubated with respective *hop solution* (Citra/El Dorado, Saaz) and *beer treatment* (Hollow Body, El Cerrito) dilutions in cell culture media at 1:16(0.0625), 1:8(0.125), 1:4(0.25), 1:2(0.5), and 1:1(1.0) represented by fraction of a stock solution (x mg/ml). Cells were incubated the various concentrations of the beer and hop treatments for 72 hours. Following incubation, cell viability was quantified using the thiazolyl blue tetrazolium bromide (MTT) assay.

Cell Viability

- HT-29/SSC-25 cells (5,000 per well) were propagated in a 96-well plate and incubated for 24 hours to adhere to the wells. The freeze dried Hops Solution and Beer Treatment were both resuspended in the cell wells at the original and diluted concentrations and were incubated for 72 hours. Cell proliferation and/or inhibition was determined spectrophotometrically via a Thiazolyl Blue Tetrazolium Bromide (MTT) assay at λ=540 nm wavelength.

Total Carbohydrate Assay

- Total carbohydrates was determined by the phenol-sulfuric acid method with glucose as the standard. The extracts were weighed and vortexed in water (1 mg/ml). Samples were diluted and combined with an 80% phenol solution. Sulfuric acid was then added and samples were held at room temperature for 10 min. Absorbance of Solutions were read at 490 nm.

Residual Sugar Test

- Residual sugar was determined by the Lane-Eynon method with glucose as the standard. The extracts were weighed and vortexed in water (20 mg and 4.7mg hops solution per ml). Samples were diluted with water and used to titrate a Fehling's solution containing Cu2+ to the equivalence point (clear solution).

Total Phenolic Content

- The total phenolic content (TPC) was determined by the Folin-Ciocalteu method using a spectrophotometry in a 96-well plate, where gallic acid was used as a standard. The diluted beer or hops sample extracts and standard were transferred at a volume of 2 µL to wells containing 158 µL of water and 10 µL of Folin-Ciocalteu's reagent. After 5 min, 30 µL of a sodium carbonate solution was added. The plates were then allowed to stand at room temperature for 2 hours before absorbance at 620 nm was measured against water blanks. The TPC was expressed as gallic acid equivalents (GAE) in mg/mg extract. The concentration of polyphenols in samples were derived from a standard curve of gallic acid ranging from 50-500 µg/mL.

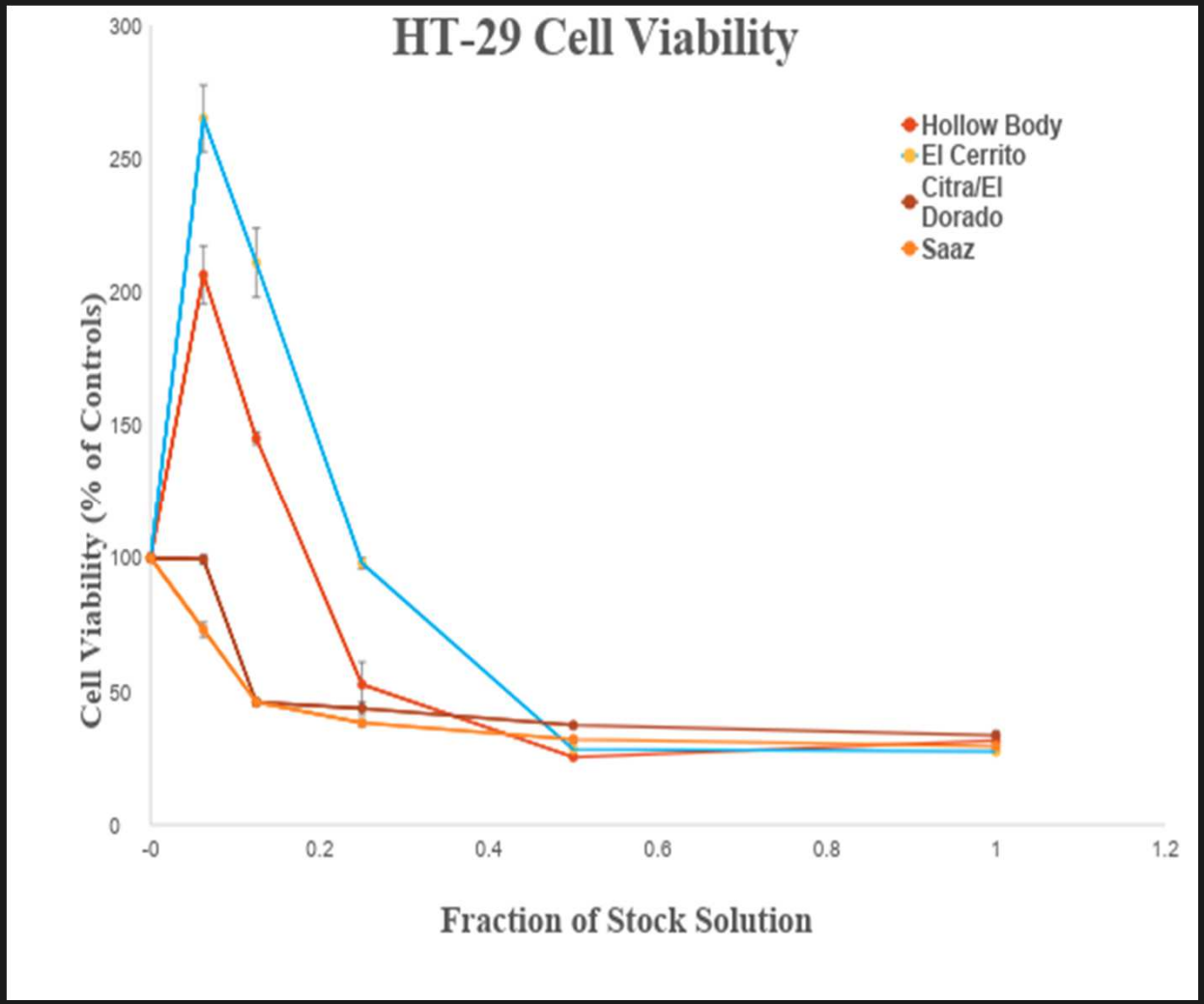
Antioxidant Capacity

- Antioxidant activity was determined by the Oxygen Radical Absorbance Capacity (ORAC) assay. The beer or hops extracts were measured out and vortexed in water (1g/10mL). Samples were then combined with 5.3 nM fluorescein in a 75 mM phosphate buffer (pH 7.4). Samples were equilibrated for 15 min at 37°C followed by the addition of 18.75 mM 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH). Fluorescence signal (485 nm excitation, 520 nm emission) intensities were monitored until baseline and peak areas were integrated. A standard curve generated from trolox peak areas was used to quantify the antioxidant capacities of the samples

Data Analysis

HT-29
Colon Cancer

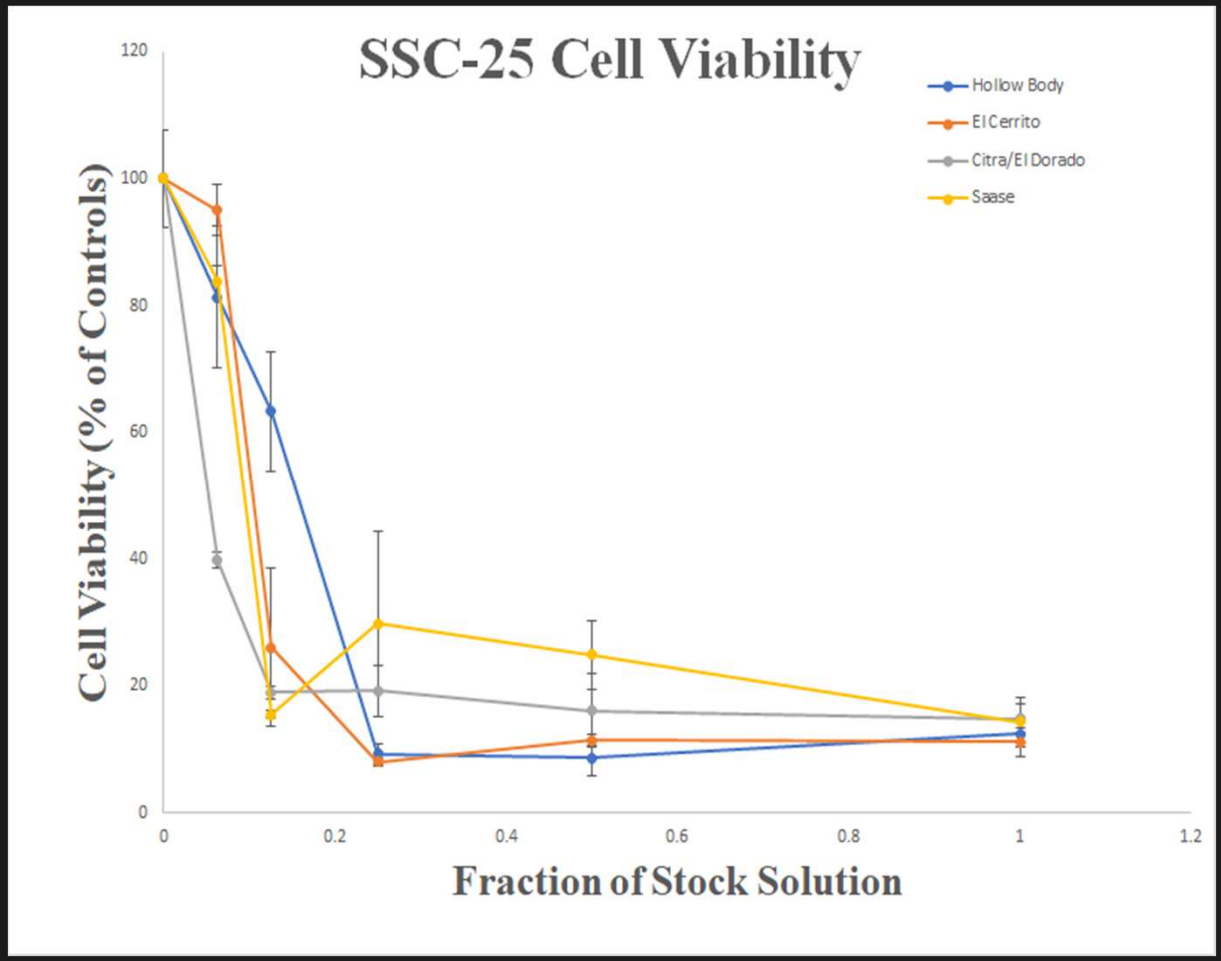
HT-29 cells suspended in dilutions of **1:16, 1:8, 1:4, 1:2, and 1:1**. Control is expressed as hundred percent growth, cell viability. At low concentrations, the beer treatments stimulated proliferation of the cell line by 206.4% (Hollow Body) and 265.0% (El Cerrito). However, as the solutions approached original concentration, inhibition of cell proliferation was observed. At all concentrations, the hops solutions inhibited cell proliferation. Specifically, cell growth was inhibited to 33.5% (Citra/El Dorado) and 29.5% (Saaz) at the original brew concentration.



Results are shown as means ± SEM (n = 3).

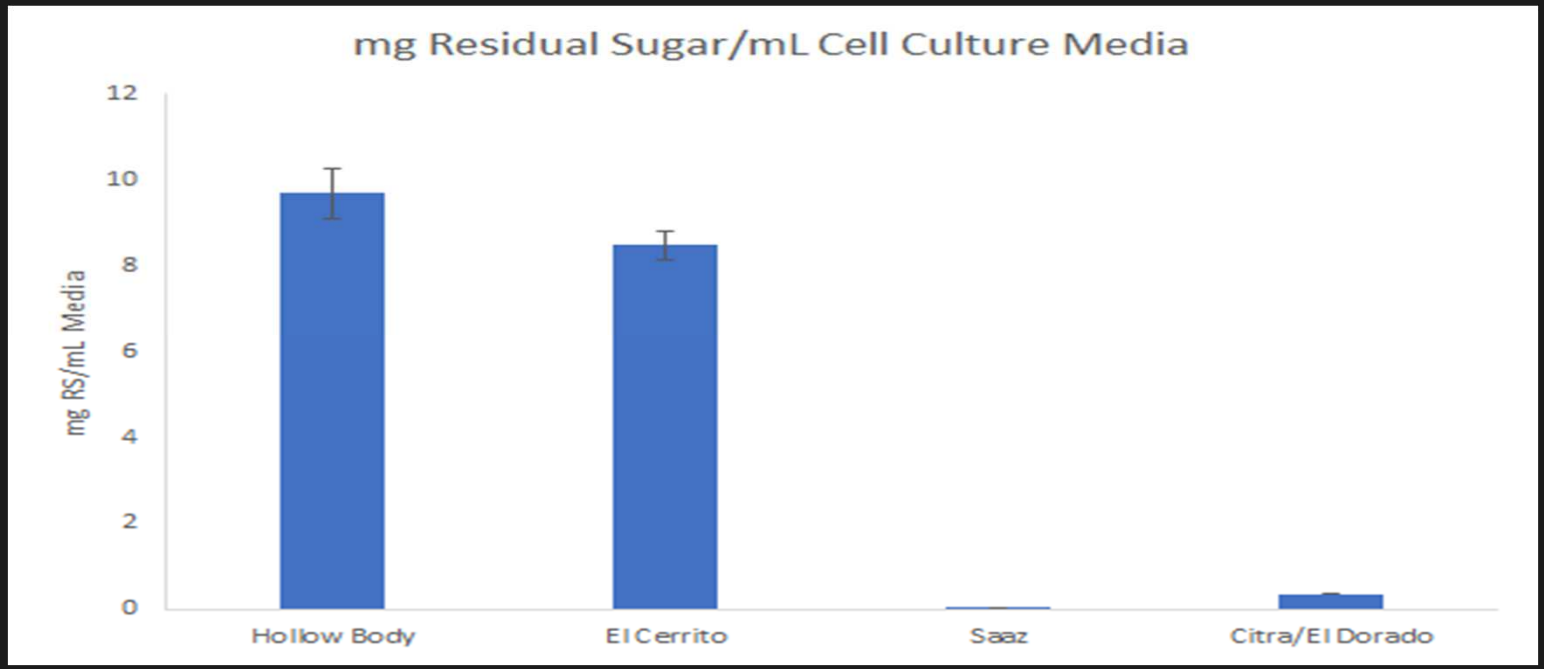
SSC-25
Oral Tongue Cancer

Cell Viability of SSC-25 squamous carcinoma cells suspended with respective hop solution (Citra/El Dorado, Saaz) and beer (Hollow Body, El Cerrito) treatment dilutions at **1:16, 1:8, 1:4, 1:2, and 1:1** represented by fraction of a stock solution. Control is expressed as hundred percent growth cell viability. The results for the beer treatments of the SSC-25 cancer line showed immediate significant inhibitory effects to the growth of the cell line, unlike the HT-29 line which had some proliferation.



Results are shown as means ± SEM (n = 2-3).

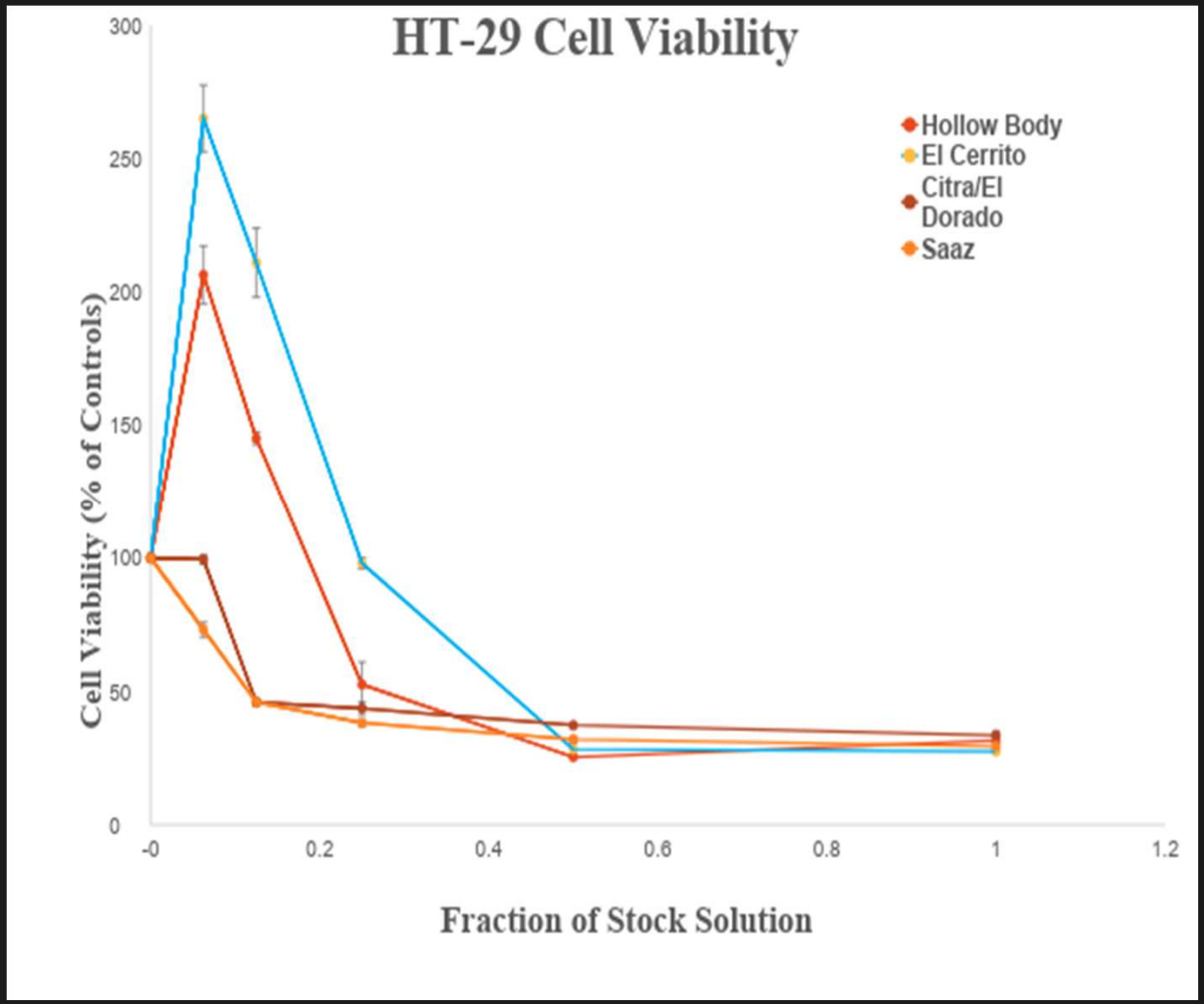
Residual Sugar Test



- Residual sugar content of beer and hops extracts. Results are shown as means ± SEM (n = 3).

HT-29
Colon Cancer

HT-29 cells suspended in dilutions of **1:16, 1:8, 1:4, 1:2, and 1:1**. Control is expressed as hundred percent growth, cell viability. At low concentrations, the beer treatments stimulated proliferation of the cell line by 206.4% (Hollow Body) and 265.0% (El Cerrito). However, as the solutions approached original concentration, inhibition of cell proliferation was observed. At all concentrations, the hops solutions inhibited cell proliferation. Specifically, cell growth was inhibited to 33.5% (Citra/El Dorado) and 29.5% (Saaz) at the original brew concentration.



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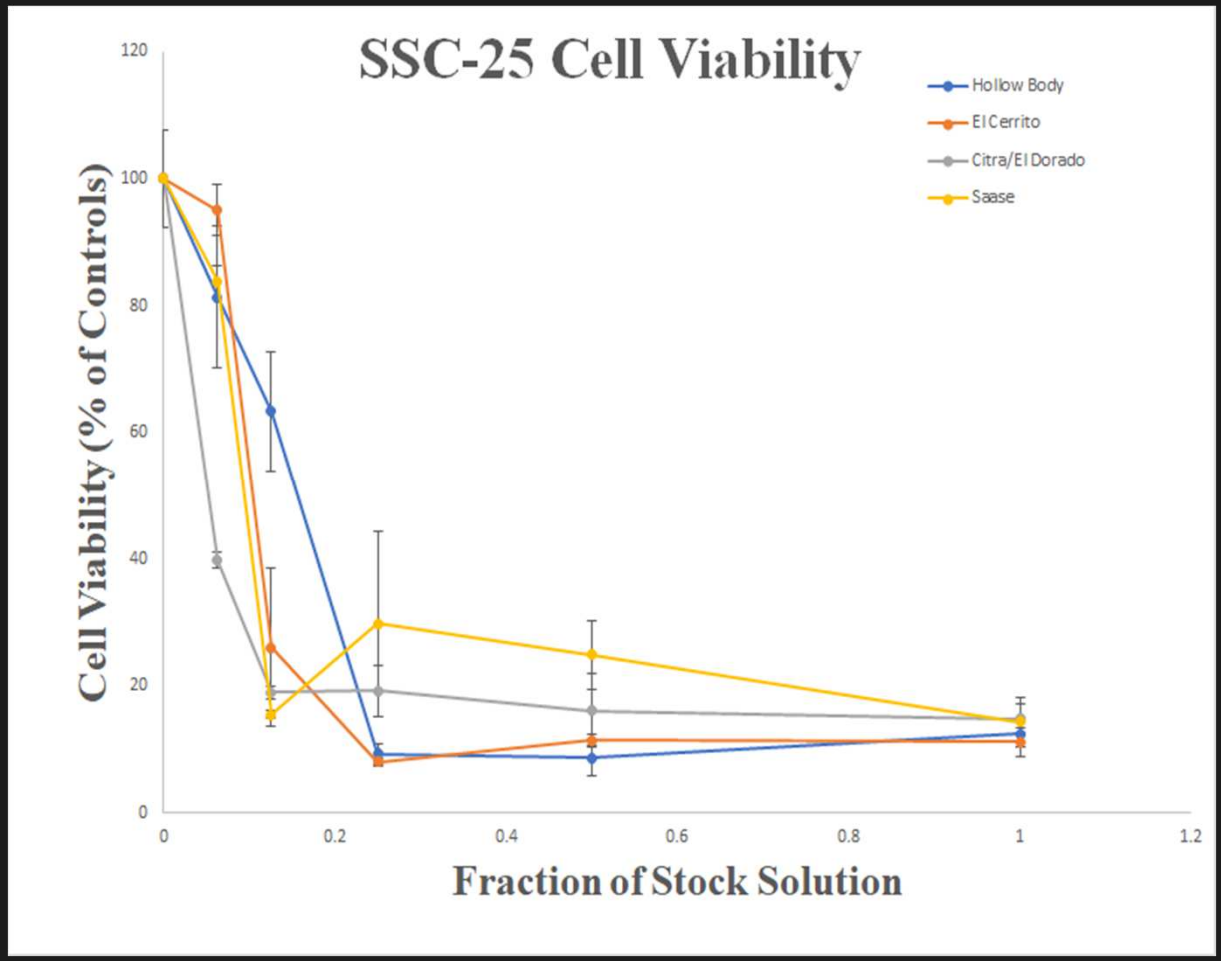
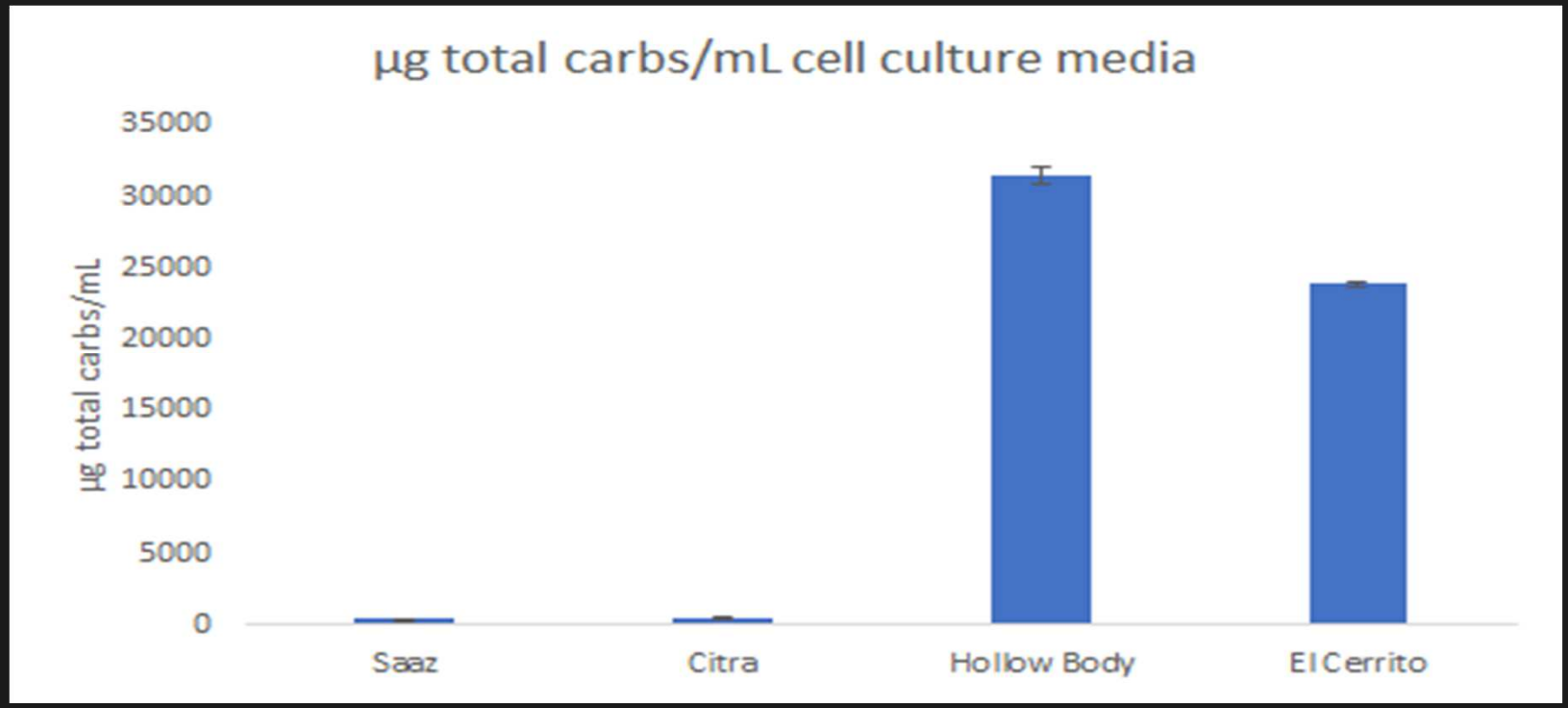


Results are shown as means ± SEM (n = 2-3).

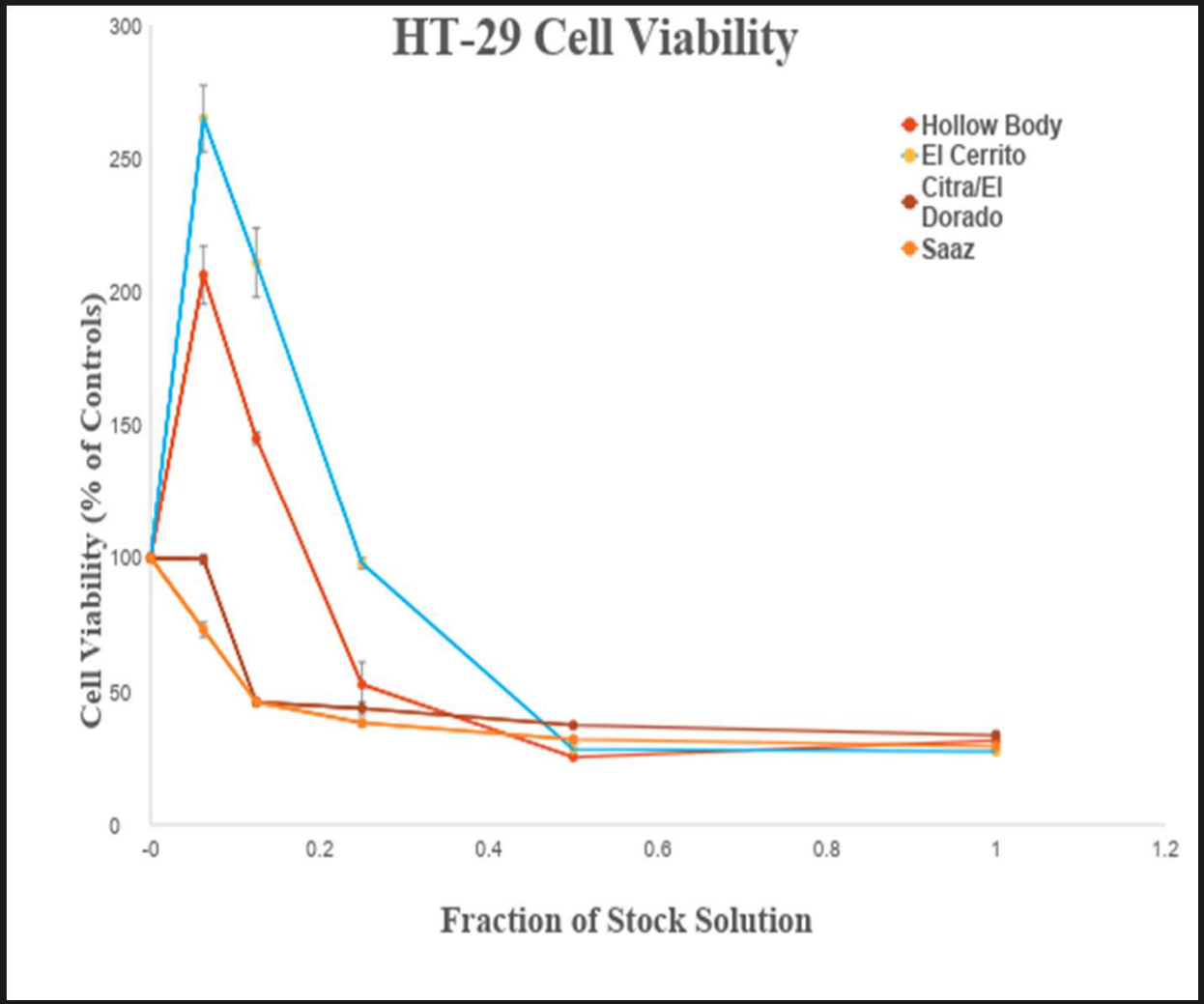
Total Carbohydrate Assay



- Total carbohydrate content of beer and hops extracts. Results are shown as means ± SEM (n = 3).

HT-29
Colon Cancer

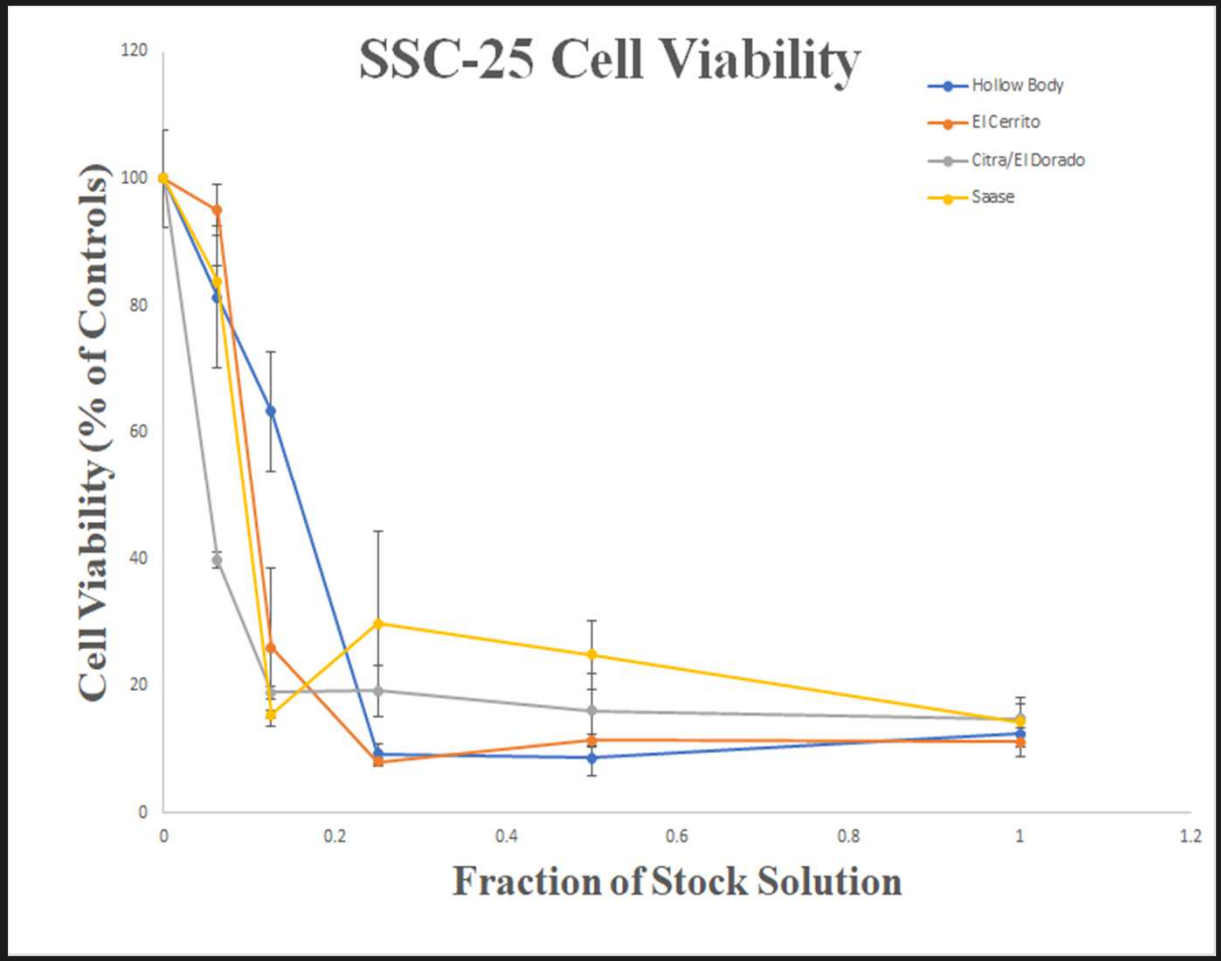
HT-29 cells suspended in dilutions of **1:16, 1:8, 1:4, 1:2, and 1:1**. Control is expressed as hundred percent growth, cell viability. At low concentrations, the beer treatments stimulated proliferation of the cell line by 206.4% (Hollow Body) and 265.0% (El Cerrito). However, as the solutions approached original concentration, inhibition of cell proliferation was observed. At all concentrations, the hops solutions inhibited cell proliferation. Specifically, cell growth was inhibited to 33.5% (Citra/El Dorado) and 29.5% (Saaz) at the original brew concentration.



Results are shown as means ± SEM (n = 3).

SSC-25
Oral Tongue Cancer

Cell Viability of SSC-25 squamous carcinoma cells suspended with respective hop solution (Citra/El Dorado, Saaz) and beer (Hollow Body, El Cerrito) treatment dilutions at **1:16, 1:8, 1:4, 1:2, and 1:1** represented by fraction of a stock solution. Control is expressed as hundred percent growth cell viability. The results for the beer treatments of the SSC-25 cancer line showed immediate significant inhibitory effects to the growth of the cell line, unlike the HT-29 line which had some proliferation.



Results are shown as means ± SEM (n = 2-3).

Total Phenolic Content & Antioxidant Capacity

Extract	µg Total Phenols/ml Cell Culture Media
Hollow Body	686.6 ± 63.4
El Cerrito	302.1 ± 43.0
Saaz	68.6 ± 3.6
Citra/El Dorado	23.4 ± 0.5

- Total phenol content of beer and hops extracts. Results are shown as means ± SEM (n = 3).

Extract	µmol Trolox Equivalents/ml Cell Culture Media
Hollow Body	18.6 ± 1.4
El Cerrito	12.9 ± 0.7
Saaz	5.8 ± 0.1
Citra/El Dorado	5.5 ± 0.8

- Antioxidant capacity of beer and hops extracts. Results are shown as means ± SEM (n = 3).

Conclusion

We conclude that the proliferative effects of the beer treatments resulted from high levels of carbohydrates such as simple sugars. Additionally, the anti-proliferative effects of the hops treatments potentially resulted from bioactive molecules such as polyphenols and alpha-humulene. In future experiments, alpha and beta acids from the hops used to make the brew will be isolated into their individual components, which will be used to treat varying cancer cell types at differing concentrations.

Additional and Future Work

Work In Progress

- Collaboration with the Food Science Department at Pennsylvania State to determine the alpha and beta acid contents; is humulone, lupulone, etc.; via Liquid Chromatography Mass Spectrometry (LCMS)
  - This will aid in explaining the bioactive properties of hops that inhibit the growth of cancerous cell lines.

Special Acknowledgements

- We would like to thank the Research Council of the University (RCU) at CSU Bakersfield, CSUB-LSAMP at CSU Bakersfield, and the CSUB Department of Chemistry & Biochemistry for facilitating this resources necessary to conduct this research.

- Additionally, we would like to thank ***Temblor Brewing Co***. and Brew-master Mike Lahti for providing the beer sample, hops, and expertise used in the brewing of the respective beer.

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