Toxicological Assessment of Medicinal Herbs to Identify Adverse Effects on Eukaryotic Cells

Abstract

Herbs are widely used as an alternative medicine without regard to adequate scientific data concerning efficacy. Cytotoxicity of herbs against different cell lines varies widely at various concentrations. This study aims to find optimal concentrations of common herbs which inhibit the prokaryotic cells but don't inhibit eukaryotic cells during short-term exposure. Yeast and E. coli k-12 bacteria cultures were exposed to ethanolic herbal extracts and respective positive controls for 48 hrs. MTS assay was used to capture absorbance readings of the microplate wells. Cytotoxicity of basil and oolong tea against bacterial cells jumped from an insignificant value to 60% and 33% respectively when their concentrations were raised from 5% to 25%. Both herbs exhibited negligible cytotoxicity against yeast cells at all concentrations. When oregano's concentration was raised from 5% to 25%, its cytotoxicity against yeast cells increased from 16% to 98% whereas its cytotoxicity against bacterial cells increased from 17% to 85%. At 20% and above optimal concentrations, basil and oolong tea had high efficacy in inhibiting the prokaryotic cells while showing statistically insignificant ($p > 0.05$) cytotoxicity against eukaryotic cells. Whereas oregano exhibited statistically significant ($p < 0.05$) efficacy against prokaryotic and eukaryotic cells at concentrations higher than 5%.

Introduction

Medicinal herbs are widely used across the world for treating various medical and dental infections based on previous experiences and common hearsay without any regard to safety, proper diagnosis, and prescription from medical and/or dental practitioners. Moreover, there is renewed interest in the scientific community for therapeutic or prophylactic use of herbal plant extracts as complementary and alternative medicine to synthetic drugs which have few major disadvantages such as serious side effects including the alteration of oral microbiota, antimicrobial resistance, and

toxicity among others (Karimi, 2015). Based on results of *in vitro* experiments, many available studies concluded antibacterial and antifungal efficacy of medicinal herbs for treating oral health problems such as cariogenic, periodontitis, and candidiasis dental infections (Kumar, 2013) but relatively little scientific data are available to demonstrate convincingly their mechanism of actions, toxicity and resulting side effects on humans, which warrants serious research in this area to generate the required evidence (Cruz, 2017). To conduct *in vitro* preliminary research, yeast is a model organism to study human biology and disease because of such similarities as having a nucleus containing DNA, division of cells. Most metabolic and cellular pathways thought to occur in humans can be studied in yeast. This enables scientists to not only study human biology through yeast but also test drugs on yeast cells containing the functional equivalent of mutated human genes to see if the drugs can restore normal function (Botstein, 1997).

Keeping the focus of this research project on the efficacy of herbs in treating bacterial infections and assessment of their toxicological impact on host cells leads one to these testable questions: Could phytochemical screening of medicinal herbs help identify underlying bioactive compounds responsible for specifically treating bacterial infections to better understand their mechanism of actions? Could *in vitro* cytotoxicity analysis (using eukaryotic and prokaryotes cultured cells) help determine the potential toxicity of medicinal herbs and/or underlying bioactive compounds to better understand their possible side effects and optimal dosages? Based on these testable questions, the hypothesis of this research project is that there exists an optimal (minimal) concentration of commonly used antibacterial herbs Ocimum sanctum, oolong tea, and Origanum vulgare which kill the prokaryotes (bacteria) cells but doesn't kill the eukaryotic (yeast) cells during short-term exposure (48 hours).

Specific Aims

The specific research goals of this project are to find the toxicity of medicinal herbs (*Ocimum sanctum*, oolong tea, *and Origanum vulgare*), having known efficacy against bacterial microorganisms, to verify the potential pharmacological effects on eukaryotic cells. In simple words, the research goal is to find the lowest concentration of the herbal extracts that kills the bacteria but doesn't kill the yeast cells.

Significance and Innovation

Medicinal herbs are widely used all over the world but not many credible scientific studies exist for evaluating their toxicity and resulting side effects on humans, which warrants serious research in this area to generate the required evidence. This research at hand will help classify widely used antibacterial and antifungal medicinal herbs and their potential pharmacological effects on human and animal tissues. Though herbal medicine may be less potent than synthetic drugs, synthetic drugs have a few major well-known disadvantages such as serious side effects including the alteration of oral microbiota, antimicrobial resistance, and toxicity among others; this clearly justifies evaluating herbal medicine as an alternative in medical and dental treatments. The medical field for the most part has already become over-dependent on synthetic chemical-based drugs bringing numerous side effects to the patients. As a viable alternative, medicinal herbs provide new opportunities to break overreliance on synthetic chemical-based drugs which could be easily avoided for minuscule non-life-threatening medical issues. Despite a large number of people using alternative medicine treatments including medicinal herbs, relatively little scientific data are available to demonstrate convincingly whether or not a particular treatment is safe, beneficial, helpful, or leads to a positive outcome (Obidike, 2013). Depending on the toxicological assessment of medicinal herbs**,** It would be far easier and practical to introduce medicinal herbs for the treatment of short-term infections or preventive care than for the longer-term cures in medicine where extensive research and *in vivo* clinical trials are needed before any application of drugs commercially. This research at hand is a focussed effort in the direction of classifying cytotoxicity data of widely used medicinal herbs, having high efficacy against bacterial infections, which can be put to immediate use for further *in vivo* testing.

This research in medicinal herbs has significant implications to the public at large. As per an article published in the New England Journal of Medicine, in 1990, Americans made approximately 425 million visits to providers of unconventional therapy often involving herbal medicine (Eisenberg, 1993). Nowadays, hundreds of internet sites are marketing herbal extracts that claim to treat, prevent, cure diagnose diseases. The World Health Organization estimates that 80 percent of the population of some Asian and African countries presently uses herbal medicine for some aspect of primary health

care. Pharmaceuticals are prohibitively expensive for most of the world's population, half of whom lived on less than $2 U.S. per day (Ekor, 2014). In comparison, herbal medicines can be grown from seed or gathered from nature for little or no cost.

Today, hundreds of herbal products sold in the USA are exempt from extensive preclinical efficacy and toxicity testing by the U.S. Food and Drug Administration. It is common practice that most dietary supplements often including herbal medicine are produced in mass, released to the market, and sold without the need to conduct the safety and efficacy just like the common pharmaceutical drugs. Additionally, FDA needs to provide evidence that the dietary supplements are not fit for human consumption before they can be removed from the market (Avigan, 2016). The discrepancies in the regulation of the compounds with pharmacological activity, therefore, raises challenges in the consistency and safety of herbal products currently produced and marketed in mass. However, many serious reports of overdose and resulting side effects of herbal extracts are documented. Heavy metal poisoning also occurs in many cases as a result of the use of herbal medicines.

The relentless overconfidence of people regarding the safety of herbal products without a proper safety verification system increases concerns about the potential severe harmful effect of some of these products, which warrants efforts such as this research at hand to help harmonize global standards of toxicity testing methods that can be used for herbal medicine toxicological characterization including tests for acute high-dose exposure effects, chronic low-dose toxicity tests and specific cellular, organ and system-based toxicity assays (Avigan, 2016).

This research effort is different from some other previous research attempts as it is focussed on some widely used medicinal herbs such as *Ocimum sanctum*, oolong tea, and *Origanum vulgare* which were found to be as effective as commercially available synthetic drugs against most common dental infections (Buggapati, 2016). There are hardly any credible and quality studies that have resulted in the compilation and classification of highly efficacious medicinal herbs along with detailed toxicity data which can be applied for immediate use for further *in vivo* clinical trials and for safe selection and dosage of medicinal herbs in dental treatments. There is a significant gap between traditional knowledge and trials investigating medical plants. Additionally, there are gaps in the quality control of herbal medicine products (Pelkonen, 2014). This research will fill some scientific research gaps before conducting *in vivo* clinical trials for the application of herbal medicine as a safe

alternative in medical and dental treatment. Future research paths of phytochemical screening and *in vitro* cytotoxicity analysis seem justified to fill the gaps in the existing state of research on medicinal herbs for the treatment of bacterial infections.

Approach and Data Analysis

Preparation of Herbal Extracts using Maceration technique

The maceration or cold extraction method will be used to prepare herbal extracts. Wash herbal leaves in clear water and dried until they were adequately dry to be ground. Dried leaves will be powdered separately in an electric grinder until a homogenous powder is obtained. Herbal powder purchased from vendors will also be further ground in an electric grinder. A total of 100 g of the finely powdered herb will be macerated with 100% ethanol for 3 days with frequent agitation. This process is intended to soften and break the plant's cell wall to release the soluble phytochemicals. This alcoholic decoction will be subjected to filtration with Whatman #1 filter paper to obtain a clear filtrate. This filtrate thus obtained will be reduced at a low temperature of less than 60°F to obtain a solid residue of herbal extract.

Methodology for toxicological assessment of medicinal herbs to identify adverse effects on eukaryotic cells - *in vitro* cytotoxic assay analysis using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay is a homogeneous, colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The assay is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyl phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS. MTS is bio-reduced by cells into a formazan product that is soluble in a tissue culture medium. The absorbance of the formazan product at 490 nm can be measured directly from 96-well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as

measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

For this research, baker's yeast (*Saccharomyces Cerevisiae*) was used to grow free-floating yeast cell culture in the culture medium (suspension culture). The yeast–peptone–dextrose (YPD) culture medium, composed of 2% glucose, 1% yeast extract and 2% bacto peptone, was kept incubated at an optimum temperature of 30°C for 72 hours to grow cells exponentially. Similarly, cell culture was prepared for E. coli k-12 strains using LB (Luria Broth) broth growth medium consisting of 10g/L tryptone, 10g/L NaCl and 5g/L yeast extract. After growing cells in the starting flask reached the exponential growth phase, contents were mixed well to have a uniform concentration of cells in the flask and for all the wells to have the same starting number of cells. Cell cultures in 50µl volume (culture medium) were carefully pipetted into wells (washing or cell harvesting not required with this cell assay) and were kept at a concentration of 10^5 cells/well in a 96-well microplate. The yeast cells were treated with different concentrations of 50µl volume of test plant extracts (50, 150, 250 mg/ml) against the positive control (0.25% fluconazole) and the negative control which contained only the medium (distilled water) and incubated for 48 hours at 37°C. The E. coli k-12 cells were treated with different concentrations of 50µl volume of test plant extracts (50, 150, 250 mg/ml) against the positive control (0.2% chlorhexidine) and the negative control which contained only the medium(distilled water) and incubated for 48 hours at 37°C. 20µl of the combined MTS/PMS solution was pipetted into each well of the 96 well assay plate containing 100µl of cells in culture medium. The plate was incubated for 1–4 hours at 37°C in a humidified, 5% CO2 atmosphere. Absorbance was recorded at 490 nm using an ELISA plate reader.

Data analysis and calculations: Background absorbance value was captured for wells containing the same volumes of culture medium and combined MTS/PMS solution as in the experimental wells.

The average background absorbance of these “no cell” control wells was subtracted from all other absorbance values.

1. Average the duplicate reading for each sample.
2. Subtract the culture medium background from Assay readings. This is the corrected absorbance.
3. Calculate percentage of dead cells (% cytotoxicity) with the following equation, using corrected absorbance:

% cell viability = [(As − Ab) / (Ac − Ab)] x 100

Where, As = Mean Absorbance reading of Test Extract sample

Ab= Mean absorbance of Blank (Media),

Ac= Mean absorbance of control (cells) without treatment,

% cytotoxicity (cell inhibition)= 100 − % cell viability

The IC_{50} (half maximal inhibitory concentration) values for all the plant extracts test compounds against both eukaryotic (yeast) and prokaryotes (bacteria) cells will be statistically compared with the control. Determination of IC_{50}, the concentration of compound required to inhibit 50 % cell growth, will be done by plotting a graph of log (concentration of extract) vs % cell inhibition. A line drawn from the 50 % value on the Y-axis meets the curve and interpolates to the X-axis. The X-axis value gives the log (concentration of the compound). The antilog of that value gives the IC_{50} value.

Statistical Analysis:

1. A one-way ANOVA test was used to compare the means of all the herbal extracts samples along with controls based on 3 trials. For this, the experimental data were expressed as mean ± SD, the significance of difference among the various treated groups and positive and negative control groups was analyzed by means of one-way ANOVA, and the level of

significance was set at $p < 0.05$.

2. After this, Tukey's posthoc statistics test will be used to compare the means of all the cell treatments. If there was a statistically significant difference of means that were found using a one-way Anova test, then Tukey's posthoc statistics test was used to exactly find the herbal extracts where those differences lie.

Risk and Safety issues

S. cerevisiae and E. coli k-12 are BSL1 organisms, and all work will thus be performed in Princeton High School Biology department laboratory which is a BSL1 facility, with appropriate personal protective equipment. All the laboratory activities will be conducted according to BSL1 procedures. During the creation and use of these protocols, all ASM biosafety guidelines will be followed.

None of the chemicals used in this research are listed as hazardous substances or mixtures according to SDS information provided by the manufacturers hence standard microbiology lab precautions should suffice. The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit used for cytotoxicity analysis, Brewer's yeast (Saccharomyces cerevisiae) and E.coli k-12 strains, used in this planned research are not considered hazardous substance or mixture according to US Regulation 29 CFR 1910.1200. Herbs to be tested in this research are commonly used as food products all over the world and are not known to have any risk associated.

https://www.asm.org/index.php/educators/laboratory-safety-guidelines

https://www.osha.gov/Publications/laboratory/OSHA3404laboratory-safety-guidance.pdf

https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PD

Hazardous Chemicals and Biological Agents Method of Disposal:

All items to be discarded after the research lab, such as culture tubes, culture plates, swabs, toothpicks, wipes, disposable transfer needles, and gloves, will be placed in a biohazard autoclave bag and autoclaved 30 to 40 minutes at 121° C at 20 pounds of pressure. In the absence of autoclave and when not working with pathogenic microorganisms, the materials will be covered with a 10% bleach solution and allowed to soak for at least 1 to 2 hours.

Summary

Given the insufficiency of formal scientific data for herbal products, this study is a significant positive step towards filling the knowledge gap in terms of chemical composition classification combined with safety data (toxicological data) of widely used medicinal herbs. Above all, the hypothesis of this study provides researchers a direct platform to effectively compare adverse effects of highly efficacious medicinal herbs and commonly used synthetic drugs on eukaryotic (yeast) cells which are similar to human cells; this is one of the essential elements in making evidence-based decisions for consideration of medicinal herbs as alternative medicine. Finally, this inexpensive research in medicinal herbs has significant implications to the public health care systems as most users of medicinal herbs use these products without any regard to safety, proper diagnosis, and prescription from medical and/or dental practitioners.

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