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Combating drug resistance - Comparison of the antibiotic effect of *Hydrastis canadensis* extract and pure Berberine via Minimum Inhibitory Concentration assay

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Abstract

Herbal medicines are a melee of complex organic chemicals, making it difficult to ascertain their direct mechanism of action. In contrast to mainstream pharmaceuticals, it is argued that herbal medicines are effective because of multiple constituents working synergistically. The complexity of herbal medicines may give them advantages over simpler pharmaceuticals in combating antibiotic resistant microbes, but these advantages can be difficult to quantitate. Popular literature frequently espouses the healing properties of herbal medicines, but many of these claims are not scientifically supported. Many gains could be realized in public health and medicine if more research was aimed at validating / disproving commonly used remedies. “Home remedies” though scientifically unsupported may still be viable treatments for certain diseases. Goldenseal (*Hydrastis canadensis*) is commonly used as an herbal therapy to treat bacterial infections, particularly of the upper respiratory tract. In an attempt to provide an organized investigation of weakly supported remedies, this research shows that extracts from Goldenseal have a greater antibiotic effect than the alkaloid berberine, which is thought to be its primary active compound. Minimal Inhibitory Concentration assays were performed with *Staphylococcus aureus* and found that the MIC of Goldenseal extract is over 15 times lower than the MIC of pure berberine. The increased bactericidal effect of the Goldenseal extract suggests synergistic effects with other compounds in the extract. Elucidation of the synergistic elements of Goldenseal extract and their mechanisms of action would be useful in creating novel methods of decreasing bacterial resistance to antibiotics.

Introduction

Modern antibiotics struggle to check the rise of drug resistant infections, and new methods are needed to combat resistant species of bacteria. As microbes develop increased levels of resistance, the medical
community must have either new, often more toxic drugs, or creatively combat the microbial modes of resistance by developing methods sufficiently different from the approaches already undertaken. One approach is to target the very agencies by which bacteria are resistant to drugs. Efflux pumps such as the ones in the major facilitator superfamily are one mechanism bacteria use to evade toxins (Marger, 1993). Inhibiting efflux pump activity may cause drugs to be retained in the cells longer, presenting the opportunity for them to have a higher potential for lethality. Ironically, some antibiotics have been shown to induce transcription of efflux systems leading directly to increased bacterial resistance (Morita, 2006). Due to these complications, it would be beneficial to identify antimicrobial compounds that target the activity of efflux systems directly, reducing the likelihood of contributing to drug resistance.

Berberine, a secondary metabolite compound found in *Hydrastis canadensis* (Goldenseal), has been shown to have antibiotic properties (Gentry, 1998; Hwang, 2003; Knight, 1999; Mahady 2003; Scazzocchio, 2001). These properties appear more pronounced in leaf or root extracts when compared to pure berberine (Ettefagh, 2011). This increased bioactivity has been suggested to be due to contemporary substances working synergistically with berberine (Gentry, 1998; Hwang, 2003; Knight, 1999; Mahady 2003; Scazzocchio, 2001). Specifically, these substances have been shown to inhibit bacterial multidrug resistant efflux pumps such as the NorA pump (Stermitz, 2000). Efflux pump inhibition allows berberine to remain in the cell longer, potentially increasing its bactericidal effect (Ibid).

This study verifies a difference in antibacterial effect between pure berberine and whole Goldenseal extracts that has been previously documented (Junio, 2011). A difference indicates that other compounds than berberine alone are working to inhibit bacterial growth; such as inhibiting the
bacteria’s drug efflux response. Whole plant extracts having an increased antibiotic effect suggests new avenues of combating drug resistant bacterial species.

**Hypothesis**

Whole root extracts (defined in procedure) from Goldenseal will have a larger antibiotic effect on Staphylococcus aureus than berberine alone as measured by 96 well plate MIC assay.

**Materials and Methods**

**Extraction**

The extracts were prepared by soaking liquid nitrogen powdered plant material for 24 hours in 5ml EtOH per 1g plant material. Alcohol extracts were separated from remaining solids by suction filtration.

**Detection of Berberine in Extract**

The presence of berberine was detected via reverse phase HPLC with an Agilent technologies Eclipse Plus C-18 column (4.6x150mm, 5µm) on an Agilent machine. The run was isocratic with 0.75 ml/min with 70% 25mM ammonium formate pH 3.8 and 30% was 0.1% triethylamine with a column temperature of 40 deg C, run for 13 minutes. Detection was performed at 230nm.

**MIC Assay**

Preparing 96 well plate

100ul of Mueller Hinton Broth was placed in each well of the plate. 100ul of extract to be tested was placed in wells A2-A6. No extract was placed in the first well or last well because the first column (A1-
H1) was the positive control (broth with inoculum only) and the final column (A12-H12) served as the negative control (broth only, no inoculum) respectively.

Two fold serial dilutions were performed with a 100ul multichannel pipet with 5 tips. 100ul of extract from the first row was pipetted into the second row and aspirated a few times. Then 100ul was pipetted from the second row to the third row and aspirated. This process was repeated to the last row and then carried over to the second half of the plate.

In the same manner, a control plate was prepared with Berberine sulfate standard at 1mg/ml initial concentration.

Inoculation

Initial broth to be tested was prepared from a 24 hour liquid culture.

Initial broth culture absorbance was read at 600nm and diluted with MHB until an optical density matching 0.5 McFarland standard was achieved. 2ml of diluted culture was placed into 38ml sterile water and vortexed. This dilution was used for inoculation. A 10ul multichannel pipette with 8 tips was used to place 10ul of inoculum in each well, moving through columns instead of rows (A1-H1, A2-H2, etc. Excluding final column as negative control).

Plate Count Confirmation of bacterial concentration

Within 15 minutes, plate count confirmation was performed by taking 10ul of inoculum placed into 10ml of sterile saline and vortexed, then 100ul per plate was pipetted onto five MHA plates. Plates were spread with cell spreader and allowed to dry before inverting and incubating.

Incubation

96 well plates were covered with autoclaved tin foil and incubated at 23°C for 16-24 hours.
MIC Determination

Plates were read with plate reader at 600nm and replicates were averaged. Minimum inhibitory concentration was defined as the concentration in the first well that showed no significant difference in turbidity from the control as suggested by error bars of 68% confidence on a bar graph.

Results

Berberine concentration in the Goldenseal root extracts (0.255 mg/mL) was determined from a standard curve constructed from HPLC analysis of a concentration series of pure berberine. Peak area and berberine concentration were found to be strongly linearly correlated \((r^2 = 0.9999)\). Peak height and berberine concentration were also very strongly correlated, but by a second order polynomial relationship. The linear relationship between peak area and berberine concentration was used to construct the standard curve in Figure 1.
Minimum inhibitory concentration assays were performed for both berberine and Goldenseal whole root extract. Minimum inhibitory dilution was determined as the lowest concentration treatment to have no significant difference in absorbance before and after incubation (Figure 2). Dilution factor of the minimum inhibitory dilution and original berberine concentration were used to determine the minimum inhibitory concentration. MIC's were compared to berberine concentration. MIC of berberine in the standard (berberine alone) was 0.0156 mg/mL. MIC of berberine in the Goldenseal root extract was 0.000996 mg/mL.

**Figure 1.** Standard curve in the form of a linear regression showing correlation of berberine concentration and peak area from HPLC chromatograms, N = 8 separate concentrations. Equation of the line $y = 0.1071x + 1.0707$. R² = 0.9999
The MIC of Goldenseal whole root extract is 15.7 times lower than that of pure berberine alone (Figure 3). HPLC and MIC data demonstrated that substantially less berberine is required in the Goldenseal whole root extract in order to have the same inhibitory effect as pure berberine alone.

**Figure 2.** Difference in absorbance before and after incubation at 23°C for 16 hours. Minimum inhibitory dilution determined by the most dilute treatment where no there is significant difference in absorbance post incubation. In this case dilution 8 was the minimum inhibitory dilution. Error bars represent standard error.
Discussion

Berberine has wide ranging mechanisms of lethality. The mechanisms for the bactericidal effect of berberine include: inhibiting DNA duplication, RNA transcription, and protein biosynthesis; influencing or inhibiting enzyme activities; destructing the bacterial cell surface structure and resulting in Ca$^{2+}$ and K$^{+}$ released from cells (Pei-ji, et al.). All of berberine’s targets are necessary for cell survival, which prohibits bacteria from developing resistance by simply mutating a lack of dependence on the berberine target.

The more than ten-fold difference in berberine concentration necessary to achieve inhibition strongly suggests the presence of another active compound in Goldenseal root extract. Berberine must not be the only antimicrobial present. Likely, Goldenseal contains other antibiotic compounds, or more interestingly, compounds which act synergistically with berberine.
This study tested only Goldenseal whole root extracts. Ettefagh et. all found that aerial portions of Goldenseal have a more pronounce synergistic effect. Synergy is observed in root extracts but is substantially increased in extracts of aerial plant portions according to Ettefagh. Berberine is present in highest concentrations in the roots of Goldenseal, but the leaves have more synergistic compounds (Ettefagh et al). Further work in this area should include HPLC fractionation on the constituents of aerial extracts. Fractions should be tested for synergistic effects and then their chemical identities elucidated. The flavonoids sideroxylin, 8-desmethyl-sideroxylin and 6-desmethyl-sideroxylin have already been shown to synergistically enhance the action of berberine (Junio et al). After such chemical elucidation, studies could aim to determine the mechanism of action by examining constituents’ effects on the Nor A efflux pump of Staphylococcus aureus. Ethidium bromide efflux inhibition has been observed due to aerial Goldenseal extracts, but not for Staphylococcus aureus derivatives with Nor A deleted (Ettefagh et al).

It is possible that a compound found primarily in aerial portions of the Goldenseal plant directly inhibits the bacteria’s method of removing the antibiotic berberine from the cytosol. This would be an important discovery because it is known that drug resistant bacteria upregulate their efflux pumps (Brown et al). Understanding of the synergistic mechanism might give insight on how to design antibiotics in ways that combat resistance.

Current pharmacological methodology is devoted to the production of highly specified single action drugs. Goldenseal, if treated as a case study of natural pharmacy, might led credence to multi-mechanism approaches to drug use. Herbal remedies have long been used as broad spectrum applications, often gaining repute from individuals of low intellectual esteem. Goldenseal is showing us that the multi-mechanism action against disease is a valuable approach. Further formal study should be
done on herbal remedies. Coupled with the intelligence of the scientific method, herbal remedies, and lessons learned from herbal remedies, could revolutionize the way drugs are developed and deployed.

Bibliography


