Evaluation of the Anti-\textit{Yersinia} Activity of Botanicals Used During the Black Plague

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Abstract

**Background:** During the fourteenth century, the Black Plague ravaged through Europe. At this time, records described using specific botanical formulations, the “Four Thieves” recipe and Nostradamus’ “Rose Petal Lozenges”, as successful therapies for preventing plague-associated bacterial infections.

**Purpose:** This study describes the characterization of the antibacterial activity associated with botanical extractions prepared from these medieval formulations against \textit{Yersinia pseudotuberculosis}, a related species of \textit{Y. pestis}, the causative agent of the Plague.

**Methods:** Ethanol and acetic acid extracts were prepared from 16 botanicals that may have been used in masks or formulated into tinctures to protect medieval populations from contracting the Plague. These extracts were tested in a dose-dependent manner related to inhibition of the growth of \textit{Y. pseudotuberculosis}.

**Results and Conclusions:** The results suggest which botanicals may have been useful in reducing infection during the historically detrimental pandemic of \textit{Y. pestis}. With a renewed and emerging threat of \textit{Yersinia}-related infections, the results provide insight into the history of botanical medicine and indicate which botanicals may act as another defensive measure against future \textit{Yersinia}-related infections.

**Keywords:** Black Death; Botanical; Plague; \textit{Yersinia Pestis}; \textit{Yersinia Pseudotuberculosis}

**Abbreviations:** CFU: Colony Forming Units

**Background**

Across Europe in the early 1300’s, the Black Death infection ended the lives of millions of humans during a plethora of epidemics [1,2]. In medieval Europe, fleas were the primary disease vector and the reservoir of the fleas were rats, especially flea-bitten rat [3]. During the 19th century, Swiss-French physician Alexandre Yersin was studying the disease, hence the name \textit{Yersinia pestis} [3]. Slightly behind him, in 1894 Japanese physician-bacteriologist Kitasato Shibasaburo was also working with the bacteria [1,3]. Ultimately, it was determined the routes of \textit{Y. pestis} infection included flea bite, or the presence of \textit{Yersinia} on a cut, wound, skin lesion, mucosa of the mouth, nose or conjunctival mucus membrane of eye [1,3]. Recent research suggests \textit{Y. pestis} may be carried by more than 200 species of rodents all over the world (except Australia and Antarctica) and at least 80 species of flea [1]. The disease has two primary forms, bubonic and pneumonic plague. Bubonic occurs typically after the bacteria invades the body following contact with inflected fleas leading to the onset of painful swelling of lymph nodes and lymph glands days to weeks after exposure [3,4]. Pneumonic is the deadliest and most contagious. It affects the lungs with victim developing severe pneumonia-like symptoms including cough, chest pain, difficulty breathing [1,3]. Pneumonic symptoms will present quickly with death occurring as rapidly as 24 hours post onset [1,3].

Metanalyses on the Plague during the 1300’s revealed two acclaimed recipes, both very similar [5,6], and another attributed to Nostradamus, that may have been used in a mask or formulated into tinctures to protect the population from contracting the deadly illness [7]. During the epidemic of \textit{Y. pestis}, tales are told of a facial mask containing herbs being used to house herbs near the
nose to protect people from getting the infection. Some peddlers drafted cure-alls to make money during these epidemics, and others reported actual cures. In the Tale of the Four Thieves, it was said that 4 men repeatedly robbed the homes of the dead, but never contracted the illness themselves. Historical investigation indicates the tale originated in either Marseilles or Toulouse [7] which is corroborated by a sizeable plague outbreak during 1720 in Marseilles [3]. According to their tale, a mother of the thieves was an herbalist that concocted an herbal vinegar recipe. She instructed them to soak a handkerchief in the herbal extract and wear it over their face so they could safely continue robbing houses [7]. The herbal vinegar recipe was used to rub on the medieval and hands as an insect repellent, to consume orally, and to carry in a small jar to inhale as vapors [3,7]. The recipe became known as the “Four Thieves” recipe. French aromatherapy doctor, Jean Valnet, has two “Four Thieves” recipes in his book [5-7]. He claims his original recipe was revealed by corpse robbers who were caught red-handed in the area around Toulouse between 1628 and 1631 [7].

Another treatment purportedly used for the plague, was developed by the prominent French physician, Nostradamus, also known as Michel de Nostredame [8]. He survived the plague outbreak (living from 1503 to 1566) and treated many others, supposedly with his “Rose Petal Lozenges” formulation [7].

The goal of this project was to investigate how the individual botanicals present in these two formulations may inhibit the replication of Yersinia bacteria when extracted in ethanol and vinegar-based tinctures. These extraction solutions were selected in an attempt to make tinctures in a similar fashion as those possibly used historically by medieval apothecaries. The genus Yersinia includes 11 species, three of which are human pathogens: Y. pestis, Y. enterocolitica, and Y. pseudotuberculosis [4]. Y. pestis evolved from parent strain Y. pseudotuberculosis [3], both of which are gram-negative bacteria [1]. Since Y. pestis is classified as a Biosafety Level 3 agent, preliminary experiments performed this study utilized Y. pseudotuberculosis as a model organism to investigate the antibacterial properties of the botanicals described in the “Four Thieves” and “Rose Petal Lozenge” formulations. Our results support the antibacterial properties of several of these botanicals against Y. pseudotuberculosis and supports the need for future studies to investigate the antibacterial activity against Y. pestis to support the efficacy of these botanical blends in inhibiting Y. pestis infections during the 1300s.

Methods

Bacteria source

The bacterial culture of Y. pseudotuberculosis (ATCC 6904), strain NCTC 2476, was obtained from American Type Culture Collection (Manassas, VA).

Botanical extract preparation

Plant material obtained from Starwest Botanicals and AmeriHerb was authenticated by HPLC profiling by the supplier with documentation of authenticity. All plant material was subsequently verified by qualified botanical specialists using herbal pharmacopoeia monographs and reference keys. A voucher specimen of all plant material was deposited in a repository/herbarium at the SCNM Botanical Institute. The botanical formulations, including the scientific and common names, of the botanicals used in the “Four Thieves” and Nostradamus’s “Rose Petal Lozenge” formulations are shown in Tables 1A and 1B. Sources of the botanicals and part of the plant used included: Acorus calamus (root) from Starwest Botanicals (Sacramento, CA), Inula helenium (root) from Starwest Botanicals, Marrubium vulgare (aerial) from Starwest Botanicals, Origanum majorana (leaf) from Starwest Botanicals, Rosa canina (petals) from Starwest Botanicals, Rosmarinus officinalis (leaf) from Starwest Botanicals, and Salvia officinalis (leaf) from Starwest Botanicals, Angelica pubescens (root) from AmeriHerb (Ames, IA), Artemisia absinthium (aerial) from AmeriHerb, Filipendula ulmaria (aerial) from AmeriHerb, and Juniperus communis (berry) from AmeriHerb, Allium sativum (fresh clove) from Whole Foods market (Chandler, AZ), Aquilaria agallocha (bark) from Ordwood, Vietnam, Campanula rapunculoides (aerial) from Woodbridge Nursery (Woodbridge, TAS), Cupressus sempervirens (leaf) from Moon Valley Nursery (Phoenix, AZ), and Iris florentina (root) from Nature’s Wonderland (Philadelphia, PA).

For extract preparation, the dried botanicals were ground to a fine powder, resuspended in extraction solution (1:6 wt:vol) and incubated at room temperature with constant mixing for 48 hours. The botanicals were extracted in 2.5% acetic acid (to simulate a historical vinegar solution) or 50% ethanol. The extract was filtered through 3 layers of netting cloth over a beaker, the collected liquid centrifuged for 3,000xg for 10 minutes and then 20,000xg for 20 minutes to remove plant debris. The supernatant was sterilized by filtration through a 0.2 μm filter. To standardize the botanical treatment doses, a sample of each extract was dried by rotary evaporation to determine the concentration (mg/ml) of the non-volatile constituents present/ml.

Bacterial growth inhibitory assay:

To calculate the minimum bactericidal concentration (MBC) with >99.9% killing, 18-hour cultures grown at 37 °C in Tryptic Soy Broth (TSB) (ranging from 1 to 5 x 10⁸ colony-forming units/ml (CFU/mL)) were diluted into TSB (1:1000 dilution) followed by the addition of the indicated concentrations of each botanical extract or control. Controls included TSB with only the botanical extract or vehicle (equivalent doses of 2.5% acetic acid or 50% ethanol), TSB with only the bacterial inoculum, or TSB with the bacterial inoculum and the vehicle (equivalent doses of 2.5% acetic acid or 50% ethanol). The cultures were incubated at 37 °C with aeration by continuous rotation for 24 hours. After 24 hours, each sample was then titrated by serial dilution. MBC endpoints were calculated as the lowest dilution of botanical extract with >99.9% killing (> 3-logs) after overnight incubation at 37 °C. The concentrations of the botanical extracts were tested at 50, 70, 90...
and 110 mg/ml. This concentration is the concentration of total non-volatile constituents present in each extract determined by rotary evaporation of each extract.

**Results**

Based on historical literature, two botanical recipes are most commonly discussed as being used during the Black Plague. Although the exact recipes of these botanical recipes are not known, the most consistent formulations are shown in Table 1A for the “Four Thieves” blends and the Nostradamus “Rose Petal Lozenges” shown in Table 1B. Much of the literature suggests these herbs may have been used in a solid form or extracted with a solvent [3,7]. For our assays, the herbs were extracted in acetic acid (to represent the historical use of vinegar) or ethanol (to represent historical alcohol extractions).

A:

<table>
<thead>
<tr>
<th>Four thieves blend 1*</th>
<th>Four thieves blend 2**</th>
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<tbody>
<tr>
<td>Artemisia absinthium  (Wormwood)</td>
<td>Artemisia absinthium  (Wormwood)</td>
</tr>
<tr>
<td>Filipendula ulmaria  (Meadowsweet)</td>
<td>Filipendula ulmaria  (Meadowsweet)</td>
</tr>
<tr>
<td>Angelica pubescens  (Angelica)</td>
<td>Angelica pubescens  (Angelica)</td>
</tr>
<tr>
<td>Origanum majorana  (Marjoram)</td>
<td>Origanum majorana  (Marjoram)</td>
</tr>
<tr>
<td>Salvia officinalis  (Sage)</td>
<td>Salvia officinalis  (Sage)</td>
</tr>
<tr>
<td>Allium sativum  (Garlic)</td>
<td>Allium sativum  (Garlic)</td>
</tr>
<tr>
<td>Rosmarinus officinalis  (Rosemary)</td>
<td>Rosmarinus officinalis  (Rosemary)</td>
</tr>
<tr>
<td>Marrubium vulgare  (Horehound)</td>
<td>Marrubium vulgare  (Horehound)</td>
</tr>
<tr>
<td>Campanula rapunculoides  (Campanula)</td>
<td>Inula helenium  (Elecampane)</td>
</tr>
<tr>
<td>Camphor</td>
<td>Juniperis communis  (Juniper)</td>
</tr>
</tbody>
</table>

*Jean Valnet, 20th century herbalist

**Museum of Paris, 1937**

Table 1: Historical botanical formulations purportedly used during the Black Plague. Listed are the proposed formulations for the Four Thieves blend (Part A) and the Nostradamus Rose petal pills/lozenges (Part B) used during the Black Plague outbreak of *Y. pestis*. For the Four Thieves blend, two proposed formulations are listed (Couto, 2019; McGruther, 2019). Botanical scientific and common names are shown. Camphor was not included in the antimicrobial assays since this study focused on botanicals alone.

As a control and to confirm that the ethanol and acetic acid vehicles in the extracts did not have any anti-*Yersinia* effects at the concentrations tested, a control experiment was done at the equivalent volumes for the mg/ml concentrations of the botanical extracts, but solely containing starting solutions of the 50% ethanol or 2.5% acetic acid. As compared to the untreated sample (bacteria only), both the ethanol and acetic acid vehicle solutions did not lead to any significant decrease in bacterial titers (Figure 1).

![Figure 1: Anti- *Yersinia* activity of extraction vehicles.](image)

Figure 1: Anti-*Yersinia* activity of extraction vehicles. For this study, botanical extractions were prepared in either acetic acid or ethanol. To confirm the lack of anti-*Yersinia* activity associated with these vehicles, *Y. pseudotuberculosis* cultures were untreated or treated with doses of the vehicles (acetic acid or ethanol) equivalent to the mg/ml doses of the botanical extracts used in subsequent assays. Treated cultures were titered by serial dilution and the final colony forming units (CFU)/mL recorded. Error bars represent the standard deviation from duplicate experiments.

Upon testing the anti-*Yersinia* activity of the various botanicals described in these historical blends, several were found to be efficacious. However, the method of extraction, whether prepared with ethanol or acetic acid, did seem to influence the antibacterial activity. As shown in Figure 2, several botanical extracts demonstrated antibacterial activity when extracted in both ethanol or acetic acid. The botanicals, *Marrubium vulgare*, *Origanum majorana*, and *Filipendula ulmaria*, were found to be effective when extracted in either ethanol and acetic acid. *M. vulgare* demonstrated a MBC (> 99.9% reduction) at 90 mg/ml when extracted in acetic acid and a reduction in bacterial titers most effectively with the acetic acid extract with over a 5-log reduction at 110 mg/mL compared to a 2-log reduction at the same dose as the ethanol extraction. *O. majorana* was one of the most effective botanicals tested, having a MBC at <50 mg/ml when extracted.

B:

<table>
<thead>
<tr>
<th>Nostradamus blend</th>
</tr>
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<tbody>
<tr>
<td>Rosa canina  (Dog rose)</td>
</tr>
<tr>
<td>Cupressus sempervirens  (Green cypress)</td>
</tr>
<tr>
<td>Iris florentina  (Iris of Florence)</td>
</tr>
<tr>
<td>Allium sativum  (Garlic)</td>
</tr>
<tr>
<td>Acorus calamus  (Calamus)</td>
</tr>
<tr>
<td>Aquilaria agallocha  (Aloeswood)</td>
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in either acetic acid or ethanol where over a 7-log reduction was observed with the acetic acid extraction and a 3-log reduction with the ethanol extract (Figure 2). Comparatively, _F. ulmaria_ was more effective when extracted with ethanol showing a MBC at 90 mg/ml and a 5-log reduction at 110 mg/mL compared to a 2.5-log reduction at 110 mg/mL of the acetic acid extract. The conserved activity of these botanical extracts when prepared in acetic acid or ethanol may suggest the presence of different active constituents or active constituents which are soluble in both extraction solutions.

**Figure 2:** Botanicals with anti- _Yersinia_ activity when extracted in either ethanol or acetic acid. _Y. pseudotuberculosis_ cultures were untreated or treated with the indicated doses (mg/ml) of botanicals extracted in acetic acid or ethanol. Treated cultures were titered by serial dilution and the final CFU/mL recorded. Figure represents those botanicals that demonstrated anti- _Yersinia_ activity when extracted in either ethanol or acetic acid. Error bars represent the standard deviation from duplicate experiments.

Alternatively, two botanical extractions were found to have anti- _Yersinia_ activity only when extracted in acetic acid. These botanicals included _Allium sativum_ and _Angelica pubescens_ (Figure 3). As shown in Figure 3, _A. sativum_ extracted in acetic acid demonstrated a dose dependent inhibition in _Yersinia_ growth with a MBC at 70 mg/mL and over a 7-log reduction at the highest dose tested (110 mg/mL). When _A. sativum_ was extracted in ethanol, no reduction in bacterial growth was observed (Figure 3). Similarly, _A. pubescens_ extracted in acetic acid gave a MBC of <70 mg/mL with a reduction in _Yersinia_ growth by over 4-logs, while the ethanol extract had no antimicrobial activity at all doses tested (Figure 3). These results suggest that an active anti- _Yersinia_ constituent was present in both _A. sativum_ and _A. pubescens_, but this compound was soluble only in acetic acid, and not ethanol.

**Figure 3:** Botanicals with anti- _Yersinia_ activity when extracted in acetic acid alone. _Y. pseudotuberculosis_ cultures were untreated or treated with the indicated doses (mg/ml) of botanicals extracted in acetic acid or ethanol. Treated cultures were titered by serial dilution and the final CFU/mL recorded. Figure represents those botanicals that demonstrated anti- _Yersinia_ activity when extracted in acetic acid, with no substantial activity when extracted in ethanol. Error bars represent the standard deviation from duplicate experiments.

On the other hand, several botanicals were found to have anti- _Yersinia_ activity only when extracted in ethanol. These botanicals included _Salvia officinalis_, _Rosmarinus officinalis_, _Artemisia absinthium_, _Cupressus sempervirens_, and _Iris florentina_ (Figure 4). Of these botanicals, the ethanol extract of _S. officinalis_ showed the greatest activity with a MBC of 70 mg/mL and a 7-log inhibition at both 90 mg/mL and 110 mg/mL (Figure 4). Only minor activity was observed with the acetic acid extract of _S. officinalis_ at the highest dose tested (Figure 4). _R. officinalis_ was also highly antimicrobial with a dose dependent reduction of _Yersinia_ growth with the ethanol tincture resulting in a MBC at 90 mg/mL (Figure 4). Again, only minor activity was observed with the acetic acid extract of _R. officinalis_. The ethanolic extracts of _I. florentina_, _A. absinthium_ and _C. sempervirens_ demonstrated anti- _Yersinia_ activity as well, but only at the highest concentrations tested (Figure 4). These botanicals had no observable activity when extracted in acetic acid.
Figure 4: Botanicals with anti-Yersinia activity when extracted in ethanol alone. Y. pseudotuberculosis cultures were untreated or treated with the indicated doses (mg/ml) of botanicals extracted in acetic acid or ethanol. Treated cultures were titered by serial dilution and the final CFU/mL recorded. Figure represents those botanicals that demonstrated anti-Yersinia activity when extracted in ethanol, with no substantial activity when extracted in acetic acid. Error bars represent the standard deviation from duplicate experiments.

Of the sixteen botanicals evaluated, I. helenium, J. communis, C. rapunculoides, A. calamus, R. canina, and A. agallocha were found to have no substantial anti-Yersinia activity when extracted in either ethanol or acetic acid (Figure 5). Historically, these botanicals may have been present in the formulations described not to provide antibacterial activity, but potentially for other roles.

Figure 5: Botanicals with no observable anti-Yersinia activity. Y. pseudotuberculosis cultures were untreated or treated with the indicated doses (mg/ml) of botanicals extracted in acetic acid or ethanol. Treated cultures were titered by serial dilution and the final CFU/mL recorded. Figure represents those botanicals that demonstrated no anti-Yersinia activity when extracted in ethanol or acetic acid. Error bars represent the standard deviation from duplicate experiments.

Discussion

Y. pestis outbreaks have occurred within the human population for hundreds of years. Recently, in 1994, the Indian cities of Surat (Gujarat) and Beed (Maharashtra) had 150 pneumonic cases and 600 bubonic cases from which 50-60 people died [3]. Between November to December of 2014, a plague outbreak occurred in Madagascar leading to 60 deaths [3]. In July of 2014, the City of Yemen in the province of Gansu in China reported one human death from Y. pestis following contact with an infected marmot [3] and there are typically human and animal epidemics each year in China [2,4]. According to the CDC, there are still up to 17 confirmed cases of bubonic plague in the US each year, and as recently as 2015, as many as 4 deaths [9]. Worldwide as recently as 2015 there were 783 cases of plague causing 126 deaths [3]. The current standard of treatment for this disease is a broad antibiotic regime which can decrease chances of death from 50-90% to 10-20% [1,3]. Some antibiotics used in this regimen include streptomycin, gentamicin, tetracycline, doxycycline and oxytetracycline, however Y. pestis is becoming increasingly antibiotic resistant [1,3].

Historical claims report that grave robbers, Nostradamus and several medieval physicians survived the Black Death utilizing “Rose Petal Lozenges” and the “Four Thieves” blends [3,8]. Identifying the potential anti-Yersinia activity of the botanicals present in these formulations may validate the historical efficacy of these blends and may provide an alternative treatment or prophylaxis to those currently living in high risk regions. Due to Y. pestis being classified as a Biosafety Level 3 agent, these initial studies had to be performed using the model organism Y. pseudotuberculosis. From this study, of the 16 botanicals examined, 10 were found to have potential antibacterial activity against Y. pseudotuberculosis, with several having very strong activity. These results support the need for future studies to investigate the antibacterial activity against Y. pestis to validate the efficacy of these botanical blends in inhibiting Y. pestis infections during the 1300s. To date, there has been limited studies on the effectiveness of these botanicals on Yersinia and how these botanicals may have influenced and saved the lives of people during the Black Plague. As described, two extraction solutions were tested to simulate what would be most commonly available in medieval apothecary formulations. For the inefficacious botanicals, these herbs may have had other roles in the historical formulation, since it has been proposed that these herbs also may have acted as insect repellants. Additionally, the mode of action of the inefficacious botanicals could have been to act in a supportive role on the immune system rather than direct inhibition of the pathogen.

Based on historical use, botanicals can be categorized by their route of administration including oral, inhaled, topical or a combination of the three routes. This is vital to the use of our botanical extractions because of disease transmission of Yersinia pestis via flea bite to the skin or contact with the respiratory system via aerosol inhalation [1]. Historical accounts mention the use of plants from these botanical blends applied topically, orally and via
inhalation [3,10,11].

In our current study, the botanicals that demonstrated activity against *Yersinia* were separated by the extraction solution in which they performed best. These anti-*Y. pseudotuberculosis* assays were performed using crude botanical extracts, so the quantitative values are based on the concentration of non-volatile components in the extracts (mg/mL) and not the actual concentration of the active constituent(s). This should be noted due to the likelihood that the active anti-*Y. pseudotuberculosis* constituents are likely different compounds and/or at varying concentrations between the different extracts. Since many of the botanicals described in these historical formulations had different activity based on the extraction method, the results suggest that several of these extracts likely have structurally different and unique active anti-*Yersinia* constituents. When combined into the historical formulations described, these different constituents likely would produce positive synergistic effects. For the results shown, the “Four Thieves” blend of botanicals appeared to have the most potent botanicals and the broadest activity based on extraction methods. Based on this, it may be predicted that the “Four Thieves” blend was more potent related to antibacterial activity and sheds some light on how these thieves may have survived plague ridden houses of the dead.

Conclusions

The goal of this study was to investigate the potential antibacterial activity of ancient botanical formulations, the “Four Thieves” and “Rose Petal Lozenge” formulations, reported used during the 1300’s for the prevention of the Black Plague. Our results, using a related and model bacterium, *Yersinia pseudotuberculosis*, suggested that the “Four Thieves” blend especially had very potent antibacterial activity and may shed some historical validation on how thieves using this formulation may have survived being exposed to the bacteria in plague ridden houses. Life threatening infections with *Yersinia pestis* are still prevalent worldwide, especially in China [1,12]. Some of the antibiotics used against *Y. pestis* are becoming ineffective due to resistance [1]. In this study, various botanical extracts were found to inhibit the growth of *Y. pseudotuberculosis*, a related species to *Y. pestis* [1,3,4]. Based on our results, future studies are warranted to test the antibacterial activity against *Y. pestis* where these results could support a potential new source of antibacterial therapeutics for the treatment of *Y. pestis* infections. These therapies could be beneficial as crude botanical extracts or offer a source for the isolation of novel pharmaceuticals. In a recent issue of Naturopathic Doctor News & Review, the World Health Organization’s definition of Anti-Microbial Resistance was discussed, calling to action for naturopathic medicine to utilize tools against the next pandemic where resources are scarce and the threat to the population is legitimate [13]. Utilizing botanical medicine against *Yersinia* may offer a legitimate way to move forward toward reaching the goals of the Global Action Plan on Antimicrobial Resistance that the World Health Organization proposes [13].

Declarations

Authors contributions: Conceived and designed the experiments: JOL AS LKH EN. Performed the experiments: AS LKH AG GJ JOL. Analyzed the data: AS LKH JOL. Contributed reagents/materials/analysis tools: JOL EN. Wrote the paper: LKH JOL, with contributions from AS. All authors have read and approved the manuscript.

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Data availability: No additional information is supplied as a supplementary file. Additional questions or information may be obtained by contact the Corresponding author, Jeffrey Langland.

References