

# Antimicrobial Efficacy of Four Thieves Vinegar Against Pneumonia-Associated Respiratory Pathogens: A Sustainable and Edible Disinfectant Approach

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**Abstract:** The aim of this study was to assess the antimicrobial efficacy of different Four Thieves Vinegar (FTV) variants, including annual, monthly and commercial FTV, against pneumonia-associated respiratory pathogens (PARPs) isolates. The well diffusion agar method was used to test the antimicrobial activity against 23 PARPs isolates such as *Staphylococcus aureus* (3 isolates), *Acinetobacter baumannii* (9 isolates), *Klebsiella pneumoniae* (6 isolates), and *Pseudomonas aeruginosa* (5 isolates). FTV was prepared by fermenting apple cider vinegar with selected several medicinal and aromatic herbs such as sage, rosemary, cinnamon, mint and lavender and others in a 3:2 ratio of apple cider vinegar to water under controlled conditions for monthly and annual incubation periods. Samples were tested in pure (100%) and 50% diluted forms. Among the variants tested, annual FTV showed the strongest inhibitory effect. The inhibition zones ranged from 19.52 mm to 16.70 mm for *K. pneumoniae* and 19.65 mm to 14.71 mm for *A. baumannii*. In contrast, monthly FTV and Apple-V showed moderate antimicrobial activity, while Commercial FTV showed the lowest efficacy, indicating that traditional fermentation enhances antimicrobial potency. The pure (100%) FTV samples generally showed larger inhibition zones than the 50% diluted samples, confirming the concentration-dependent efficacy of FTV. The antimicrobial effects varied with fermentation time and vinegar composition, with longer fermentation times correlating with stronger inhibitory activity. FTV showed strong antimicrobial potential against PARPs, making it a natural alternative to chemical disinfectants and highlighting the value of traditional methods. Further research should optimise formulations and assess stability against other hospital-acquired multidrug-resistant pathogens.

**Keywords:** Antimicrobial activity, four thieves vinegar, disinfectant, plant mixed vinegar, pneumonia-associated respiratory pathogens

## 1. Introduction

Four thieves' vinegar, a 17th century remedy from Toulouse, was thought to protect against the plague. It was made with sage, thyme, and rosemary, and highlights the potential of vinegar-based formulations to combat infections, including pneumonia (Shelton, 2019; Kelley, 2020). Throughout history, pandemics have affected human health and plants have been used as primary treatments. Herbal remedies date back to 1500 BC in Egypt and were later included in the Greek, Roman and official pharmacopoeias. They remain essential in regions where modern medicine is

limited (Akerle, 1993). Global interest in herbal medicine stems from its perceived safety, but clinical validation remains a challenge (Atanasov et al., 2015). Increased botanical research could support new therapeutic developments (Mitjà et al., 2021).

Vinegar, first produced by the Egyptians, has been used for preservation, flavouring and medicine (Li et al., 2005; Garcia, 2020). It is produced by fermentation of carbohydrates using alcohol and acetic acid bacteria. Bioactive compounds with antimicrobial and antioxidant properties are produced (De Roos and De Vuyst, 2018;

Karthikeyan et al., 2020). TSE 1880 EN 13188 defines vinegar as a two-stage fermentation product. Types include wine, fruit, cider, malt and flavoured vinegar (Dilimen et al., 2021; Anlı and Çapar, 2024). Solieri and Giudici (2009) with Giudici et al. (2015) reported that acidity standards stipulate at least 4% acetic acid in USA and 5% in Europe.

Vinegar preparations have strong antimicrobial properties and have been used to treat fever, sepsis, and nervous disorders (Ledermann-Dehnhardt, 2021). A historical remedy with *Angelica archangelica*, *Cinnamomum verum*, *Allium sativum*, *Origanum vulgare*, *Filipendula ulmaria*, *Artemisia absinthium*, and *Salvia officinalis* macerated in vinegar was applied to the skin to prevent plague, likely due to its flea-repelling and pain-relieving effects (Garcia, 2020).

Moreover, wine vinegar is a functional food with antimicrobial and antioxidant properties (Laukkanen-Ninios et al., 2014). Aromatic plants, rich in polyphenols and volatile compounds, enhance flavor and offer health benefits (Duarte and de Fátima Carrijo, 2014). Their composition varies by species and environment, and terpenes like limonene and linalool contribute to antimicrobial and antioxidant effects (Van de Vel et al., 2019; Boncan et al., 2020; Kopaczyk et al., 2020; Flores and Toldrá, 2021). However, the use of aromatic plants in wine vinegar remains underexplored, with

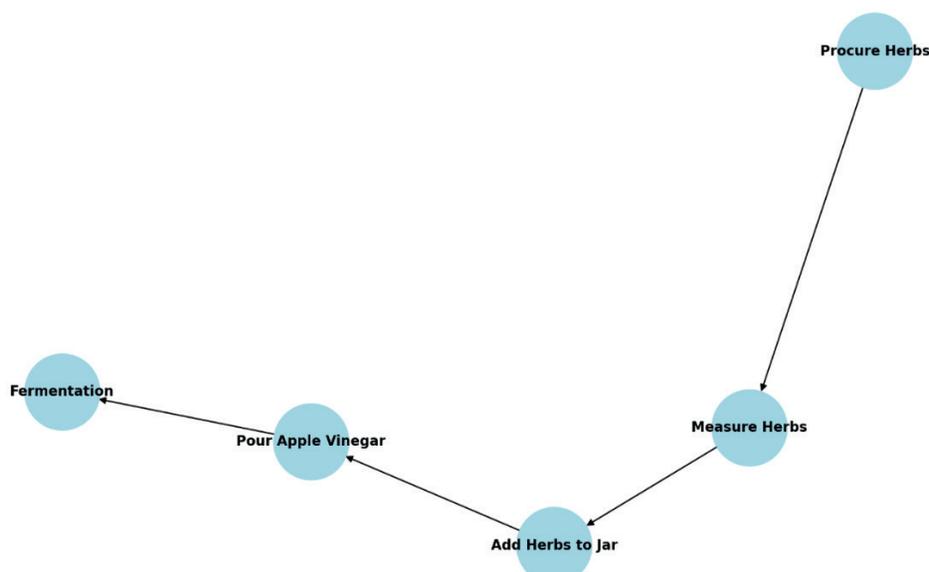
most research focusing on flavor enhancement through herbs, fruits, and vegetables (Buncic et al., 2014; Kaveh et al., 2022; Radi et al., 2023).

This study aimed to evaluate the antibacterial activity of four different Four Thieves Vinegar (FTV) formulations against pneumonia-associated respiratory pathogens (PARPs) such as *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), *Staphylococcus aureus* (*Staphylococcus aureus*), and *P. aeruginosa* to assess their potential as natural alternatives to chemical disinfectants for surface and mucosal disinfection in pneumonia prevention and management.

## 2. Materials and Methods

### 2.1. Preparation of FTV from medicinal and aromatic plants (MAPs) with apple-V

Figure 1 illustrates the process of determining the original formulation of FTVs. Preparation begins with the collection of ingredients, including apple vinegar (also known as cider vinegar), water and selected herbs. These components are mixed in a sterilised container at a 3:2 ratio of apple vinegar to water. The mixture is then covered with cheesecloth and stored in a dark, room temperature environment to ferment. After the fermentation monthly and annual period, the mixture is filtered through a fine cloth to separate the liquid. Finally, the samples are prepared in both pure



**Figure 1.** Flowchart for FTV preparation process

(annual/monthly) and 50% diluted forms, labelled and stored for further analysis. Firstly, the apples were washed and cut into small pieces. Local apples were used to make the apple-V and MAPs to FTVs (Table 1). They were then placed in a sterilized 5 L

container. A 30 g of sugar, a 50 g of salt, and a slice of bread were added to the container as a starter for the fermentation process. Then 100 mL of home-made vinegar was poured over the mixture and the container was filled with drinking water until it was

**Table 1.** Vinegar preparation: List of herbs and amounts of four thieves vinegar

Vinegar	Common name	Latin name	Amount
Apple vinegar (for 5 L)	Apple	<i>Malus domestica</i>	3000 g
	Sugar		30 g
	Salt (NaCl)		50 g
	Bread		1 slice
Four thieves vinegar for 5 L (3:2) vinegar: Water	Sage	<i>Salvia officinalis</i>	
	Rosemary	<i>Rosmarinus officinalis</i>	40 g
	Cinnamon	<i>Cinnamomum zeylanicum</i>	40 g
	Dried mint	<i>Mentha spicata</i>	40 g
	Wormwood	<i>Artemisia absinthium</i>	40 g
	Ginger	<i>Zingiber officinale</i>	40 g
	Lavender	<i>Lavandula angustifolia</i>	40 g
	Rue	<i>Ruta graveolens</i>	20 g
	Blueberry	<i>Acorus calamus</i>	40 g
	Nutmeg	<i>Myristica fragrans</i>	5 g
	Black pepper	<i>Piper nigrum</i>	40 g
	Garlic	<i>Allium sativum</i>	10 pieces

completely submerged. The container was covered with cheesecloth to allow air to circulate and stored in a dark room at ambient temperature until the apples sank to the bottom, indicating fermentation was completed. Once fermentation was complete, the mixture was strained through a fine cloth to separate the liquid from the solids, yielding the final apple vinegar product (Figure 2).

A comprehensive literature review was conducted to identify the plants traditionally used in FTV. The necessary herbs were then sourced for preparation. Selected plants were combined with apple cider vinegar. The mixture was left to macerate in a dark environment for 30 days, with daily agitation. On the 10th day, additional camphor was added. After 30 days, the vinegar was filtered through cheesecloth to remove plant residues. Market research was also conducted on FTV products sold in the Adana market, and samples were purchased. All microbiological analyses were performed at the Microbiology Laboratory of the Biotechnology Research and Application Centre of Çukurova University.

## 2.2. Preparation of concentrations

A pure sample of four thieves vinegar, which has been waiting for a one-year-old pure sample of annual-FTV, 50% and 100% diluted, sample monthly-FTV 50% and 100% diluted, homemade apple cider vinegar (apple-V) 50% and 100% diluted. The same dilution was for commercial-FTV. The pH measurements of four different vinegar pure samples were 50% diluted with distilled water.

## 2.3. Antimicrobial activity assay

This study focused on PARPs indicator microorganisms such as *P. aeruginosa* (5 isolates), *K. pneumonia* (6 isolates), and *S. aureus* (3 isolates) and *A. baumannii* (9 isolates) obtained from tracheal aspiration cultures of mechanically ventilated patients in the Reanimation Unit of Çukurova University, Faculty of Medicine (November 2016 to June 2017).

The well diffusion agar method according to Erhonyota et al. (2023) was used to evaluate the antimicrobial activity of FTVs against indicator

**Figure 2.** Preparation of FTV from apple-V and MAPs in laboratory

microorganisms. The Clinical and Laboratory Standards Institute (CLSI)-standardised Mueller-Hinton agar method was used to assess antibiotic resistance of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* and *S. aureus* isolates. The PARPs as indicator microorganisms stored below 5 °C were reactivated in tryptic soy broth (Tryptic Soy Broth, Merck 1.05459) at 37 °C for 18 hours. Mueller-Hinton agar (Merck 1.05437) was prepared by transfer of 12 mL agar (cooled to 50 °C) into 90 mm petri dishes. Fresh bacterial cultures, adjusted to 0.5 Mc-Farland density, were mixed with the agar and allowed to solidify before 6 mm diameter wells were made. Each well was filled with 100 µL of FTV extract and incubated at +4 °C for diffusion, followed by overnight incubation under optimal conditions. Moreover, the zone measurements against microorganisms were observed, the zones they formed were measured with a caliper in millimeters.

### 3. Results and Discussion

Results included antimicrobial activity of different vinegar types, concentrations, and fermentation times against a range of pathogens, annual-FTV 100% highest showed antimicrobial efficacy against all strains tested, with larger zones of inhibition compared to monthly-FTV, commercial-FTV, and apple-V in Table 2.

**Table 2.** Comparison of the pH of vinegars

	Vinegars pH measurement
Control*	3.93
Annual-FTV	4.13
Monthly-FTV	4.34
Commercial-FTV	4.29

\*: During the pH measurements, it was observed that the ambient temperature was approximately 23±2

The pH measurements show that apple cider vinegar had the lowest pH (3.93), making it the most acidic and potentially the most effective antimicrobial. However, the means show clear differences, with the monthly FTV having the highest pH (the least acidic) and apple cider vinegar the lowest pH (the most acidic). These differences likely result from variations in fermentation time and preparation methods. All measurements were taken at a stable ambient temperature of 23±2 °C. The antibacterial effect of acetic acid against various pathogenic bacteria has gained recognition, particularly in the context of food safety, due to its preservative properties (Zapašnik, et al., 2022). Acetic acid formed by acetic acid bacteria is acknowledged as safe by the Food and Drug Administration (FDA) and has been approved by several regulatory bodies, including The European Commission, The Food and Agriculture

Organization (FAO), and the World Health Organization (WHO), for use as a food additive (Bangar et al., 2022).

The antimicrobial activity of different types of vinegar, including annual-FTV, monthly-FTV, commercial-FTV and controls (at 100% and 50% concentrations), was evaluated against *K. pneumoniae* and others. Inhibition zone diameters (in mm) showed that annual-FTV had the highest antimicrobial activity, with inhibition zones of 18.37 to 24.26 mm at 100% concentration and 14.74 to 19.12 mm at 50% concentration. Monthly-FTV also showed a moderate level of activity, with inhibition zones ranging from 13.96 to 18.27 mm at 100% concentration and from 10.52 to 12.71 mm at 50% concentration. Commercial-FTV was relatively less active. The inhibition zones ranged from 10.41 to 13.67 mm. The control samples showed the lowest activity with inhibition zones ranging from 9.28 to 15.32 mm (Table 3).

These results suggest that the antimicrobial efficacy varies significantly between the different types of vinegar, with the annual-FTV showing a superior activity against *K. pneumoniae*. MAPs are rich in terpenes, organic compounds contributing to their aroma, flavor, and defense mechanisms, classified into monoterpenes, sesquiterpenes, diterpenes, triterpenes, and tetraterpenes (Ninkuu et al., 2021). Terpenes play key roles in attracting pollinators, deterring herbivores, and allelopathy, while also exhibiting antimicrobial, anti-inflammatory, and antioxidant properties valuable in medicine and food preservation (Xu et al., 2022). For example, monoterpenes like limonene and linalool enhance scents in cosmetics, and sesquiterpenes contribute to food flavor and aroma. Volatile compounds in rosemary include verbenone,  $\alpha$ -thujene, bornyl acetate, and camphor thyme contain p-cymene and elderflowers are rich in tocotrienol, linalool oxide, and  $\alpha$ -terpineol. Polyphenols in these plants, such as rosmarinic acid, carnosic acid, rutin, and chlorogenic acid, provide health benefits, extend shelf life, and improve sensory attributes (Krapac et al., 2024).

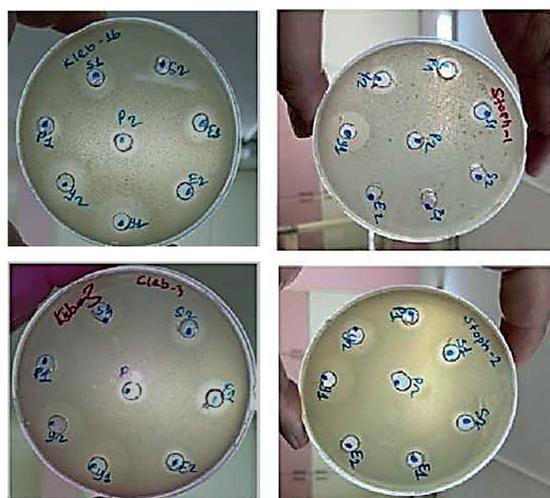
*Pseudomonas* isolates showed variation depending on the vinegar type and concentration in Table 3. Annual-FTV demonstrated the highest and the most consistent efficacy, with inhibition zones ranging from 13.81 to 19.08 mm at 100% concentration, outperforming both monthly-FTV and commercial-FTV. Monthly-FTV 100% vinegar showed moderate activity, particularly against *P. aeruginosa*-7 and *P. aeruginosa*-11, with zones up to 15.2 mm, though it failed to inhibit *P. aeruginosa*-4. In comparison, commercial-FTV displayed limited antimicrobial activity, with

**Table 3.** Inhibition zone diameter (mm) of all vinegar formulation on PARPs

Microorganisms (PARPs)	Annual-FTV		Monthly-FTV		Apple-V		Commercial-FTV	
	100	50	100	50	100	50	100	50
<i>Klebsiella pneumoniae</i> isolates								
<i>K. pneumoniae</i> -2	18.37	15.93	14.46	10.88	12.86	9.37	12.57	10.19
<i>K. pneumoniae</i> -3	20.08	17.21	13.96	11.49	15.32	11.44	13.67	10.75
<i>K. pneumoniae</i> -4	24.26	18.00	18.27	11.98	14.74	11.40	Nd	11.16
<i>K. pneumoniae</i> -6	18.04	15.17	11.72	10.66	12.26	9.53	12.05	17.75
<i>K. pneumoniae</i> -16	22.02	19.12	14.95	12.71	14.06	10.65	13.13	10.47
<i>K. pneumoniae</i> -9	14.34	14.74	16.41	10.52	11.92	9.28	10.41	9.04
<i>Pseudomonas aeruginosa</i> isolates								
<i>P. aeruginosa</i> -1	13.81	12.44	13.43	Nd	13.08	Nd	12.39	Nd
<i>P. aeruginosa</i> -4	18.08	15.36	Nd	Nd	Nd	Nd	Nd	Nd
<i>P. aeruginosa</i> -7	19.08	16.08	15.20	11.71	14.73	10.74	16.04	11.10
<i>P. aeruginosa</i> -11	14.18	13.21	10.91	Nd	9.26	Nd	Nd	Nd
<i>Acineobacter baumannii</i> isolates								
<i>A. baumannii</i> -1	19.35	14.08	9.95	Nd	9.95	Nd	9.60	8.55
<i>A. baumannii</i> -3	20.21	14.38	10.67	9.74	10.95	8.71	11.22	8.61
<i>A. baumannii</i> -5	20.10	15.77	12.88	10.86	15.05	10.08	21.40	9.95
<i>A. baumannii</i> -6	16.28	12.00	18.44	13.05	13.88	9.92	10.26	9.09
<i>A. baumannii</i> -8	19.83	15.76	13.42	10.38	13.14	10.62	12.43	9.25
<i>A. baumannii</i> -10	27.14	15.17	11.94	Nd	10.70	9.63	10.02	Nd
<i>A. baumannii</i> -20	15.66	14.77	12.08	9.71	11.74	9.72	11.47	9.39
<i>A. baumannii</i> -30	15.98	13.63	11.63	10.01	10.82	9.52	11.21	8.71
<i>A. baumannii</i> -31	22.29	17.88	16.30	12.76	16.02	11.66	15.74	12.27
<i>Staphylococcus aureus</i> isolates								
<i>S. aureus</i> -1	18.81	12.30	11.01	10.15	11.35	Nd	11.92	Nd
<i>S. aureus</i> -2	20.41	16.73	12.67	10.59	12.92	11.04	12.72	11.98
<i>S. aureus</i> -3	13.49	10.38	13.41	9.60	11.35	9.80	10.41	8.27

Nd: Not detected

inhibition zones not exceeding 16.04 mm and no effect on some strains, such as *P. aeruginosa*-1 at 50% concentration. Control samples exhibited the weakest activity overall, with inhibition zones ranging from 9.26 to 14.73 mm (Figure 3).



**Figure 3.** FTV generally exhibited an inhibitory effect on *K. pneumoniae* and *S. aureus*

Among the variants, Y1 (annual FTV at 50% concentration) and Y2 (annual FTV at 100% concentration) showed the highest inhibition zone diameters. Monthly-FTV: S1 and S2, Commercial-FTV: P1 and P2, Apple-V: E1 and E2

These results suggest that annual-FTV is more effective against *P. aeruginosa* strains compared to other vinegar types. Research conducted by Sakhare et al. (1999) explores the efficacy of acetic acid as an antimicrobial agent in various types of meats, such as poultry, beef, and pork, aimed at prolonging shelf life and effectively de-contaminating pathogens like *Salmonella* spp. and *E. coli*. Lactic acid bacteria members contributes significantly to food safety and shelf life by suppressing pathogenic and spoilage bacteria. They are also essential in fermentation, enhancing food preservation, safety, flavor, and aroma (Abiola et al., 2022; Bhattacharya et al., 2022; Mgomi et al., 2023).

The *S. aureus*, among the tested vinegar types, annual-FTV 100% demonstrated the highest antimicrobial activity, with inhibition zones ranging from 13.49 to 20.41 mm at 100% concentration and 10.38 to 16.73 mm at 50%. This was particularly evident in *S. aureus* 2, where the inhibition zone reached 20.41 mm at 100%, significantly surpassing the other vinegar types. Monthly-FTV exhibited moderate activity, with inhibition zones ranging from 11.01 to 13.41 mm at 100% concentration and 9.6 to 10.59 mm at 50%. Although it was effective, its performance was generally lower than annual-FTV, especially against *S. aureus*-2 (Table 3).

Commercial-FTV displayed limited antimicrobial activity, with inhibition zones not exceeding 12.72 mm at 100% concentration and showing no inhibition against *S. aureus*-1 at 50%. Its overall effectiveness was the weakest among the tested vinegar types. Control samples also showed minimal activity, with inhibition zones ranging from 9.8 to 12.92 mm at 100% concentration and 11.04 mm at 50%, further emphasizing the enhanced efficacy of annual-FTV in comparison to the other treatments (Table 3). Potential activity of kombucha beverages against pathogenic enteric bacterial infection. Kombucha has become a preferred functional beverage due to its several health benefits (Sanwal et al., 2023).

Apple vinegar exhibited moderate antimicrobial activity, showing some effectiveness against *K. pneumoniae*, *S. aureus*, *P. aeruginosa*, and *A. baumannii*. However, traditional long-fermented vinegars, particularly the annual-FTV, demonstrated significantly superior antimicrobial performance compared to apple vinegar and other fermentation types. This enhanced activity is likely attributed to the extended fermentation process, which optimizes the extraction and concentration of bioactive compounds. In contrast, the monthly-fermented vinegar displayed lower effectiveness, with a noticeable reduction in inhibition compared to the annual-FTV. Commercially available FTV also showed relatively weaker antimicrobial properties, further highlighting the importance of the fermentation period in maximizing the vinegar's antimicrobial potential. Therefore, the prolonged fermentation period of the annual-FTV is a key factor in its enhanced antimicrobial efficacy, making it more effective than both shorter-fermented and commercially prepared vinegars. This emphasizes the significant role of fermentation time in optimizing the antimicrobial benefits of vinegar.

#### 4. Conclusions

This study shows that herb-enriched vinegar could serve as a sustainable and cost-effective alternative to synthetic disinfectants in healthcare settings. Its enhanced antimicrobial activity, particularly against PARPs and more broadly against multi-drug resistant pathogens, offers significant potential for healthcare applications. Further research is needed to optimise vinegar formulations with medicinal herbs and explore their integration into infection control protocols to combat these resistant pathogens.

#### Ethical Statement

The authors declare that ethical approval is not required for this research.

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#### Declaration of Author Contributions

Conceptualization, Material, Methodology, Investigation, Data Curation, Visualization, Writing-Original Draft Preparation, Writing-Review & Editing, *N. YILMAZ*; Data Curation, Formal Analysis, Writing-Review & Editing, *M.O. KAYA*. All authors declare that they have seen/read and approved the final version of the article ready for publication.

#### Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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