



Research Article

Efficacy of *Calendula Officinalis* as an Antibacterial Agent for *In Vitro Pseudomonas Aeruginosa*

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Abstract

Objectives: The aim of this in vitro microbial study was to evaluate the efficacy of *Calendula officinalis* tincture 60% (v/v) ethanol as an antibacterial agent for in vitro *Pseudomonas aeruginosa*.

Methods: The standardized disk diffusion method was employed. Seven pairs of Mueller–Hinton agar plates were used; one plate was the experiment and one a control. *P. aeruginosa* broth cultures were grown for 24 hours. A sterile cotton swab was soaked in *P. aeruginosa* broth culture and then streaked evenly in 3 directions over the entire surface of the agar plates to obtain a uniform inoculum. Sterile filter paper disks (0.45- μ m pore size; 5-mm diameter) were impregnated with standardized amounts of *C. officinalis* tincture 60% (v/v) ethanol. The first set of disks was impregnated with undiluted *C. officinalis* tincture 60% (v/v) ethanol, and the subsequent sets of disks were impregnated with a series of 2-fold dilutions of *C. officinalis* tincture 60% (v/v) ethanol. One disk from each set was aseptically placed on the inoculated agar surface of each plate. The plates were inverted and incubated at 37°C in ambient air for 24 hours, after which any presence of clear zones of inhibition were observed against a light background and the diameter of these zones was measured. The whole procedure was performed in triplicate. The mean activity of the 2 treatment groups was 6.88 and 6.69 mm, respectively.

Results: The antibacterial activity of *C. officinalis* tincture 60% (v/v) ethanol and the 60% ethanol control groups showed a similar pattern on in vitro *P. aeruginosa*. There was no statistically significant difference between the experimental and control groups ($p > 0.05$; t-test).

Conclusion: There was no evidence to prove the efficacy of *C. officinalis* tincture 60% (v/v) ethanol as an antibacterial agent for in vitro *P. aeruginosa*.

Keywords: Antibacterial, antibiotic resistance, *Calendula officinalis*, efficacy, in vitro, *Pseudomonas aeruginosa*

With the widespread emergence of antibiotic resistance, especially among potential nosocomial pathogens such as *Pseudomonas aeruginosa*, evaluation of alternative antimicrobial agents is important.^[1] Such increases in antibiotic resistance among *Pseudomonas* species are in line with a general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistance among many groups of bacteria.

P. aeruginosa continues to be a major cause of infections

even in western society, in part because of its high intrinsic resistance to antibiotics. Hancock has demonstrated that this intrinsic resistance arises from the combination of usually restricted outer membrane permeability and secondary resistance mechanisms such as energy-dependent multi-drug efflux and chromosomally encoded periplasmic beta-lactamase.^[2] Given this high level of natural resistance, mutational resistance to most classes of antibiotics can readily arise.

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It has been established that *P. aeruginosa* infections are the most troublesome in terms of nosocomial infections.^[3] For example, contaminated non-touch fittings in hospitals are a great hazard, especially when they are installed in risk areas such as intensive care units.^[4] Moreover, *P. aeruginosa* infections may accelerate lung disease in children, and this may result in a very high rate of mortality in pediatric wards.^[5]

Many pathogens are developing resistance to most currently used antibiotics, and there are increasingly frequent reports of pathogens that are resistant to almost all available antibiotics.^[6] Antibiotic resistance in bacteria has been linked to overuse of antibiotics in animals and humans.^[7]

Plant extracts typically contain a number of active components, some of which provide multiple benefits while some provide a mixture of benefits and side effects.^[8] *Calendula officinalis* has received limited study, although it does have antimicrobial properties, insignificant toxicity both with its one-time and chronic administration, and it is also devoid of local irritation properties.^[9] However, the inappropriate and extravagant use of *C. officinalis* can cause neurological toxicity.^[10] Specifically, there is no available research on *C. officinalis* as an antibacterial agent against the most troublesome antibiotic resistant species such as *P. aeruginosa*.^[8]

Quantitative assay of antimicrobial agents by microbiological methods represents a special application of microbial inhibition. Tests have been designed so that a relationship exists between the degree of antimicrobial activity and the quantity of the antibacterial agent, that is, within certain limits of antibacterial concentration, proportionality exists between the amount of antibacterial and the degree of inhibition.^[11]

Therefore, the purpose of this study was to evaluate the efficacy of *C. officinalis* tincture 60% (v/v) ethanol as an antibacterial agent on *in vitro P. aeruginosa*. The results from this study may be of clinical significance in terms of contributing toward the development of an alternative to antibiotics.

Methods

Materials

- Mueller–Hinton agar in 100-mm petri plates (2–8°C), agar depth of 5 mm
- *C. officinalis* tincture 60% (v/v) ethanol stock 100 mL; purchased from Floro Force, Cape Town, South Africa
- Sterile cotton-tipped swabs
- Sterile Pasteur pipettes
- McFarland turbidity standard
- Sterile 5-mm filter paper disks
- Clean dry surface

Methods

The standardized simple disk diffusion method as used to evaluate the efficacy of *C. officinalis* tincture 60% (v/v) ethanol as an antibacterial agent on *in vitro P. aeruginosa*. *C. officinalis* tincture 60% (v/v) ethanol was compared with 60% ethanol as a control. The whole procedure was performed in triplicates in accordance with the National Committee for Clinical Standards (NCCLS).

Seven pairs of 100-mm petri plates were used. From each pair, one disk was used as the experiment and another as the control. Therefore, the prepared Mueller–Hinton agar were poured onto 14 plates and allowed to solidify. The agar depth was set at 5 mm per plate.

A series of two-fold dilutions of *C. officinalis* tincture 60% (v/v) ethanol were made. That is, a series of seven sets of dilutions were made (undiluted, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64). An analogous series of two-fold tube dilutions of the 60% ethanol control (final volume 1 mL) were also made.

Seven pairs of filter paper disks were set aside. One set of each pair comprised seven disks. One set of each pair was impregnated with the corresponding dilution of *C. officinalis* tincture, and the other set was impregnated with the corresponding dilution of 60% ethanol. That is, one set of the first pair of disks was impregnated with undiluted *C. officinalis* tincture 60% (v/v) ethanol, while the other set was impregnated with undiluted 60% ethanol. Similarly, one set of the second pair of disks was impregnated with the 1/2 dilution of *C. officinalis* tincture 60% (v/v) ethanol, whereas the other set was impregnated with the 1/2 strength dilution of 60% ethanol. The same procedure was followed until the first and second set of disks of the seventh pair were appropriately impregnated with the 1/64 dilution of *C. officinalis* tincture 60% (v/v) ethanol and 1/64 dilution of ethanol, respectively.

Using a sterile loop, five *P. aeruginosa* colonies of similar morphology (from overnight agar plate) were picked and placed in 3-mL phosphate-buffered saline as a diluent. This was shaken until an organism suspension equivalent to McFarland 0.5 turbidity standard was obtained.

A sterile cotton swab was dipped into the *P. aeruginosa* suspension, and excess fluid was expressed against the side of the tube. The dry surface of each Mueller–Hinton agar plate was inoculated by streaking the swab in three directions over the entire surface of the plate in order to obtain a uniform inoculum. After each plate, the swab was discarded in a battery jar containing a disinfectant solution (methylated spirit).

One of the disks that were impregnated with the undiluted *C. officinalis* tincture 60% (v/v) ethanol was aseptically

and carefully placed on the surface of one of the Mueller–Hinton agar plates of the first pair, and it was ensured that contact was made with the agar. One of the disks that was impregnated with the undiluted 60% ethanol was aseptically placed on the surface of the second Mueller–Hinton agar plate of the first pair. Similarly, one disk of the disks that was impregnated with the 1/2 strength dilution of *C. officinalis* tincture 60% (v/v) ethanol was aseptically placed on the surface of the first Mueller–Hinton agar plate of the second pair. The corresponding disk that was impregnated with the 1/2 strength dilution of ethanol was aseptically placed on the surface of the second Mueller–Hinton agar plate of the second pair. The same procedure was followed until the seventh pair of the Mueller–Hinton agar plates was reached.

The plates were then inverted, and incubated at 37°C in ambient air for 24 h. After incubation, the presence or absence of clear zones of inhibition was observed by examining each plate against a light background. With the aid of a millimeter ruler, the diameter of such zones was measured. If zones of inhibition were absent or indistinct, the plates were incubated for an additional 24 h before determining the final result. Any indistinct growth was considered as absence of antibacterial activity. Antibacterial activity was considered to be absent if the zone size was <7 mm and present if the zone size was >11 mm. Antibacterial activity was considered to be intermediate if the zone size was

7–11 mm. Intermediate antibacterial activity would warrant further investigation by the agar dilution method. The diameter of each disk was included in the measurement of the zone of inhibition.

It is important to note that the foregoing procedure was performed in triplicate. This means that a total of 42 Mueller–Hinton agar plates were used (i.e., 21 experiments and 21 controls). One disk per plate was preferred over the five disks per plate method to avoid any possibility of overlap of zones of inhibition.

Statistical Analysis

After comparing the variances using F-test, a proper student's two-tailed t-test (with equal variance) was employed to compare means between two samples (significance level: $p < 0.05$). The antibacterial activity of *C. officinalis* tincture 60% (v/v) ethanol was assessed by regression analysis (significance level: $p < 0.05$; ANOVA). All statistical analyses were conducted with Stata (Stata Corporation, 4905 Lakeway Drive, Texas 77845, USA).

Results

The results as reflected in Tables 1-4 showed that under the test conditions, *in vitro* *P. aeruginosa* showed some susceptibility to the undiluted *C. officinalis* tincture 60% (v/v) ethanol. The two-fold serial dilutions of *C. officinalis* tincture 60% (v/v) ethanol showed no antibacterial activity on *in*

Table 1. Antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* *Pseudomonas aeruginosa*

Test Treatment (First pair/group)	Dilution (Concentration)	Zone of Inhibition (mm)	Activity
Calendula officinalis			
1 st set	1 (neat)	15.5	present
2 nd set	½ (0.5)	6	absent
3 rd set	¼ (0.25)	6	absent
4 th set	1/8 (0.125)	6	absent
5 th set	1/16 (0.0625)	5.5	absent
6 th set	1/32 (0.03125)	5	absent
7 th set	1/64 (0.015625)	5	absent
	Mean=7		
Ethanol			
1 st set	1 (neat)	16	present
2 nd set	½ (0.5)	6	absent
3 rd set	¼ (0.25)	5	absent
4 th set	1/8 (0.125)	5	absent
5 th set	1/16 (0.0625)	5	absent
6 th set	1/32 (0.03125)	5	absent
7 th set	1/64 (0.015625)	5	absent
	Mean=6.7		

Table 2. Antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* *Pseudomonas aeruginosa*

Test Treatment (Second pair/group)	Dilution (Concentration)	Zone of Inhibition (mm)	Activity
Calendula officinalis			
1 st set	1 (neat)	17	present
2 nd set	½ (0.5)	5.5	absent
3 rd set	¼ (0.25)	5.5	absent
4 th set	1/8 (0.125)	5	absent
5 th set	1/16 (0.0625)	5	absent
6 th set	1/32 (0.03125)	5	absent
7 th set	1/64 (0.015625)	5	absent
	Mean=6.9		
Ethanol			
1 st set	1 (neat)	17	present
2 nd set	½ (0.5)	6	absent
3 rd set	¼ (0.25)	5.5	absent
4 th set	1/8 (0.125)	5	absent
5 th set	1/16 (0.0625)	5	absent
6 th set	1/32 (0.03125)	5	absent
7 th set	1/64 (0.015625)	5	absent
	Mean=6.9		

Table 3. Antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* *Pseudomonas aeruginosa*

Test Treatment (Third pair/group)	Dilution (Concentration)	Zone of Inhibition (mm)	Activity
Calendula officinalis			
1 st set	1 (neat)	16	present
2 nd set	½ (0.5)	6	absent
3 rd set	¼ (0.25)	5	absent
4 th set	1/8 (0.125)	5	absent
5 th set	1/16 (0.0625)	5	absent
6 th set	1/32 (0.03125)	5	absent
7 th set	1/64 (0.015625)	5	absent
	Mean=6.7		
Ethanol			
1 st set	1 (neat)	15	present
2 nd set	½ (0.5)	5	absent
3 rd set	¼ (0.25)	5	absent
4 th set	1/8 (0.125)	5	absent
5 th set	1/16 (0.0625)	5	absent
6 th set	1/32 (0.03125)	5	absent
7 th set	1/64 (0.015625)	5	absent
	Mean=6.4		

vitro *P. aeruginosa*. The results also showed that *in vitro* *P. aeruginosa* was susceptible to the undiluted 60% ethanol, and the serial two-fold dilutions of 60% ethanol diminished the antibacterial activity on *P. aeruginosa*.

Discussion

This study showed that there was no significant difference between the antibacterial activity of *C. officinalis* tincture 60% (v/v) ethanol and that of 60% ethanol control on *in vitro* *P. aeruginosa* ($p > 0.05$; t-test). Although the mean activities were 6.88 and 6.69 mm in groups 1 and 2, respectively, there was no evidence that this was more than could be expected from sampling variation ($p = 0.88$). Converting the dilution and/or the activity to logarithms did not alter the result materially. The undiluted solution alone had a significant antibacterial effect on *in vitro* *P. aeruginosa* ($p < 0.001$; ANOVA).

The lack of evidence against the null hypothesis of the treatments being equivalent did not imply any evidence that they were the same. That is, it would not be proper to claim that they have been proved to be equivalent, rather this study merely failed to prove that they were different, which could be due to the sample being small.

Given the constituents of *C. officinalis*, one would have expected *C. officinalis* tincture to have a significantly pronounced antibacterial activity than the control. In fact, a

Table 4. Mean effect of dilution on the antibacterial activity of *Calendula officinalis* tincture 60% (v/v) ethanol versus 60% ethanol on *in vitro* *Pseudomonas aeruginosa*

Test Treatment (Mean of the three pairs)	Dilution (Concentration)	Zone of Inhibition (mm)	Activity
Calendula officinalis			
1 st set	1 (neat)	16.2	present
2 nd set	½ (0.5)	5.8	absent
3 rd set	¼ (0.25)	5.5	absent
4 th set	1/8 (0.125)	5.3	absent
5 th set	1/16 (0.0625)	5.2	absent
6 th set	1/32 (0.03125)	5.0	absent
7 th set	1/64 (0.015625)	5.0	absent
	Mean=6.9		
Ethanol			
1 st set	1 (neat)	16.0	present
2 nd set	½ (0.5)	5.8	absent
3 rd set	¼ (0.25)	5.2	absent
4 th set	1/8 (0.125)	5.0	absent
5 th set	1/16 (0.0625)	5.0	absent
6 th set	1/32 (0.03125)	5.0	absent
7 th set	1/64 (0.015625)	5.0	absent
	Mean=6.7		

synergistic antibacterial activity would have been expected between the constituents of *C. officinalis* and the 60% ethanol present in the tincture.

It is possible to postulate the reasons for the limited antibacterial activity of *C. officinalis* tincture 60% (v/v) ethanol. First, the concentration of the antibacterial active ingredients of *C. officinalis* in the tincture was probably far too low or nil. Second, the stability of the *C. officinalis* components was possibly hampered by the storage conditions of the tincture and the environmental conditions under which the study was carried out. Furthermore, it has to be borne in mind that *in vitro* environments are markedly different from *in vivo* environments. Therefore, results from *in vitro* studies are not always compatible with results from *in vivo* studies. For example, a major difference between bacterial colonies and biofilms in patients (such as those on vascular, urinary, or peritoneal catheters or on orthopedic prostheses) and in aqueous environmental locations is that the antibacterial agent-containing medium to which the colonies are exposed to is static. Because of this lack of mixing, the antibacterial agent in the immediate vicinity of the colonies will rapidly be depleted, and this could be expected on apparent reduction in antibacterial susceptibility. However, even in the case of biofilms bathed in a well-mixed liquid medium, there is still a diffusive mass transfer region close to the film surface.^[12,13]

The standardized simple disk diffusion method is normally used to examine the efficacy of antibiotics in accordance with NCCLS guidelines. However, there are no guidelines for testing the antibacterial efficacy of plant extracts such as *C. officinalis* tincture. For instance, some of the guidelines that were employed in this study were those that are normally used for examining the susceptibility of enterococci to aminoglycosides. By employing the standardized disk diffusion method, the antibacterial efficacy of *C. officinalis* tincture is influenced by a complex of factors, such as diffusion rate of the tincture through the agar, the inoculum size, growth rate of the bacterial colonies, viability of the bacteria, and its susceptibility to the tincture. *P. aeruginosa* is actually a non-fastidious and rapidly growing bacterial species.^[14]

The stability of *C. officinalis* tincture at different temperatures should be taken into consideration. To illustrate this point, the stability of ozonized sunflower oil (oleozon) is documented. Oleozon is stable for up to 1 year in the temperature range -10 to $+8^{\circ}\text{C}$. Moreover, it is stable for up to 6 months at room temperature ($27-30^{\circ}\text{C}$); after this period, the antibacterial properties diminish. The pH is also stable for up to 1 year in the temperature range -10 to $+8^{\circ}\text{C}$. At 30°C , the pH is stable for up to 6 months.^[15] Therefore, there is a possibility that the efficacy of the constituents of *C. officinalis* was also influenced by factors such as temperature, pH, and concentration.

Conclusion

This study demonstrated that there was no evidence to prove the efficacy of *C. officinalis* tincture 60% (v/v) ethanol as an antibacterial on *in vitro* *P. aeruginosa*. There was no statistically significant difference between the activity of the experiment and control groups.

Based on the results of this study, it would be easy to conclude that the antibacterial properties of *C. officinalis* tincture 60% (v/v) ethanol can be attributed to the 60% ethanol present in the tincture. In retrospect, it is essential to consider the fact that the concentration of the constituents of *C. officinalis* tincture 60% (v/v) ethanol was not determined. It would be essential to determine the stability of *C. officinalis* tincture 60% (v/v) ethanol under the storage and experimental conditions. Therefore, a quality control program must be adopted to accurately examine the efficacy of *C. officinalis* tincture 60% (v/v) ethanol as an antibacterial on *in vitro* *P. aeruginosa*.

Further study is needed to determine the effects of additional factors such as inoculum size, inoculum pre-compared to post-addition of *C. officinalis* tincture 60% (v/v) ethanol, short term kill-curves, long-term survival, and

possible selection of resistant variants. Thus, since *P. aeruginosa* is a common contaminant that causes infections with various vascular catheters, medications, contact lens solutions, non-touch fittings in hospitals, and even some disinfectants, *C. officinalis* may warrant further study as an additive to topical preparations or in other areas such as harvesting of blood or blood products or even to peritoneal dialysis solutions to reduce *P. aeruginosa* contaminants.

This study should lead to the setting up of some clinical trials to compare the efficacy of *C. officinalis* with other antibacterial agents. The wide availability of *C. officinalis* could make it a competitive antibacterial agent. It will require the combined effort of both researchers in the clinical and laboratory fields to verify the usefulness of *C. officinalis* and other potential antibacterial agents and their clinical importance. Hopefully, in the near future, continued research and cooperation between clinical and laboratory researchers will bring *Pseudomonas* infection treatment protocols closer to the needs of the individual patient.

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