



1,8-CINEOLE HEALS CUTANEOUS PSEUDOMONIASIS IN GOATS: AN ANIMAL MODEL STUDY

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Summary

Saeedi, F., G. Jalilzadeh-Amin, V. Najarnezhad & A. Ownagh, 2023. 1,8-cineole heals cutaneous pseudomoniasis in goats: An animal model study. *Bulg. J. Vet. Med.*, **26**, No 4, 567–575.

Pseudomonas aeruginosa, an opportunistic bacterium is also an important pathogen in animals, resistant to various antibiotics in different ways. The purpose of the present study was to evaluate 1-8 cineole, a new antimicrobial agent derived from medicinal plants. Four male and female goats about two years of age weighing 45 kg were used. To prepare the *P. aeruginosa* inoculation site, a 30 cm² area in the right and left flank and thorax region were shaved. The inoculum was injected intradermally at five sites, with 0.4 mL in a concentration of 10⁶ CFU/mL of *P. aeruginosa* per site. After the induction of cutaneous pseudomoniasis, lesions in the right thoracic area were treated with 1-8 cineole, lesions in the left thoracic area treated with gentamicin ointment as a standard drug and lesions in the right flank were treated with glycerin daily until complete recovery. Daily administration of cineole and gentamicin for nine days improved skin lesions. Same as the untreated group (n=4), glycerin did not affect cutaneous lesions. The results of this study showed that 1,8-cineole, same as gentamicin, healed and improved cutaneous lesions in goats caused by *Pseudomonas* infection within nine days.

Key words: 1,8-cineole, cutaneous lesions, essential oils, goat, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium responsible for many infections among immunocompromised hosts, burned patients and individuals suffering from cystic fibrosis. It is an important opportunistic pathogen and has minimal nutritive requirements with great

adaptability (Yi *et al.*, 2010). *P. aeruginosa* is usually present in the environment. Its ability to readily grow, and relative resistance to chemical disinfectants make impossible eradication of this pathogen (Bannerman *et al.*, 2005). Several virulence factors were produced by *P.*

aeruginosa – haemolysin is cytotoxic for most cells and is considered the most potent toxin produced, lecithinase (phospholipase) can destroy cell membranes, and protease degrades proteins which bring about inflammatory responses and cell death (Constable *et al.*, 2016). Prolonged oral medication at therapeutic levels may result in superinfection with *P. aeruginosa*. Septicaemias, toxæmia, pleurisy, pyelonephritis, arthritis, and severe mastitis occur most commonly in all animal species. In neonate calves, *P. aeruginosa* is one of the common causes of infection and mortality. Fleece rot in small ruminants is associated with *P. aeruginosa*. Some of the identifiable occurrences of dermatitis in food animals and horses are caused by a combination of trauma and infection by *P. aeruginosa* (Constable *et al.*, 2016).

The bacterium is resistant to antimicrobials and biocides, that allows it to achieve further adaptation and infection (Bannerman *et al.*, 2010; Park *et al.*, 2014). This threatens the public health on a global scale as it reduces the effectiveness of medications and increases morbidity, mortality and health care costs. Consequently, one of the common control strategies is producing new antimicrobial drugs from medicinal plants and their essential oils in order to make their derivatives compounds more efficient. The 1,8-cineole, also known as eucalyptol, is a monoterpene present in many plant essential oils. Several studies have mentioned its antimicrobial activity (Lambert *et al.*, 2001; Oliveira *et al.*, 2010; Stojkovic *et al.*, 2011; de Sousa *et al.*, 2012). Therefore, the presence of 1,8-cineole in a large number of plants that are widely used in folk medicine, makes their antimicrobial action against the pathogenic microorganisms more pronounced.

Although some researchers have found that 1,8-cineole has antimicrobial activity against *Pseudomonas* spp (Gachkar *et al.*, 2007; Tyagi *et al.*, 2010; Azerêdo *et al.*, 2011; de Sousa *et al.*, 2012), there are no studies about its antimicrobial effect when applied in infection and lesions that clinically occurred. Here we report the investigation of an enhanced antimicrobial effect when 1,8-cineole was used in experimentally induced cutaneous pseudomoniasis.

MATERIALS AND METHODS

Test organism and 1,8-cineole

P. aeruginosa (PTCC 5027) was obtained from the microbial bank of the Mycology section in the Department of Microbiology, Faculty of Veterinary Medicine, Urmia University. Stock cultures were kept on Brain-Heart-Infusion agar (Merck, Hamburg, Germany) under refrigeration (4 °C). The inoculum, 10⁶ CFU/mL *P. aeruginosa* (PTCC 5027) used in assays, was obtained from suspensions of two colonies of bacterium from overnight cultures on Brain-Heart-Infusion agar, incubated at 37 °C for 24 h. To prepare a suspension the colonies were dissolved in 0.9% NaCl; turbidity equal to the 0.5 McFarland turbidity standard was used.

Pure 1,8-cineole (98%) was obtained from Sigma Co. (USA) through the representative of this company. Based on previous experiments, in order to stabilise and retain 1,8-cineole at the site of experimental wounds and to dilute it to the required dose, the effective substance was dissolved in glycerol. To estimate the *in vivo* effective concentration of 1,8-cineole, laboratory studies (using MIC and MBC methods) were first designed and ten times of the effective concentration observed in the culture medium was used as a therapeutic dose on animals.

Since the pure solution prepared by the company was free of infectious agents, no sterilisation was performed, but in the end, the obtained solution was sterilised by passing it through a 0.22 micron syringe filter. The sterile solution was kept in refrigerator until use.

Animal preparation

The *in vivo* study protocols were approved by the Animal Research Ethics Committee (Approval number 1395/05). The trial was carried out on goats of a native breed. The animals are considered free of other infections and other cutaneous disorders based on clinical inspection of the skin. In this study, four goats (two males and two females) with a weight of approximately 45 kg about two years of age were kept in the clinic of the Faculty of Veterinary Medicine of Urmia University. The animals were fed alfalfa twice a day and had free access to water. Two weeks before the start of the study, animals were treated against possible parasites with two broad-spectrum antiparasitic drugs, albendazole at a dose of 7.5 mg/kg body weight twice in 14 days and ivermectin at a dose of 0.2 mg/kg body weight. Also, for treatment and prevention of other possible bacterial skin diseases, and by observing the appropriate 'time interval' between antibiotic treatment and experimental induction of the disease, they were injected subcutaneously once with long-acting oxytetracycline 20% (11 mg/kg body weight). To suppress the immune system of animals, dexamethasone (2 mg/kg body weight) was injected intramuscularly daily for 4 days (Koptopoulos, 1992).

To prepare the *P. aeruginosa* inoculation site, an area of 30 cm² at each of the right and left thorax and flank were shaved. After immune system suppression,

0.4 mL of *P. aeruginosa* suspension (at a concentration of 10⁶ CFU/mL) was injected intradermally at five points at each site, approximately 2 cm apart (Hazrati *et al.*, 2010; Tanideh *et al.*, 2014). When cutaneous pseudomoniasis was induced, lesions in the right thoracic region were treated with 1.8-cineole (1250 µg/mL), lesions in the left thoracic region – with gentamicin ointment (10 million units per 100 g) as standard medicine and the lesions in the right flank – with glycerin (control locus) until complete recovery. Lesions in the left ventricular region were not treated as a negative control locus.

Evaluation of the treatment

In order to evaluate clinically the trend of skin lesions changes, the sites of *P. aeruginosa* inoculation in all four animals were photographed daily from the first day to the end of the treatment period (18th day) and information recorded in computer memory. In addition to determining the possible systemic involvement, clinical signs and vital signs were evaluated. The lesion sites in successfully treated animals were sampled and cultured again at the end of the study.

RESULTS

One day after inoculation of *P. aeruginosa* suspension, the inoculation site was swollen and red (Fig. 1A). The inoculated goats looked a little dull and lethargic. They were resting in a sternal position and did not pay much attention to the environmental stimuli. The average body temperature of these animals was 40.2 °C, but the animals had normal appetite and continued to eat. On the third day, their average body temperature was 39.5 °C.

On the fourth day, the inoculation area was swollen and red. In the centre of some of the swellings white and gray spots were seen. Small vesicle-like lesions were also observed on some swellings (Fig. 1B). On the fifth day after inoculation, the inoculation area was still swollen, red, hot and painful. Purple areas were seen in the centre of some of the swellings. The centre of some of the swellings was sunken, and scab formation following the rupture of some of them was seen (Fig. 1C). Similar lesions were observed at sixth and seventh day after inoculation of *P. aeruginosa* suspension at the inoculation site.

Daily administration of cineole for nine days following intradermal inocula-

tion of *P. aeruginosa* improved skin lesions (Fig. 1D). At the end of the ninth day, the affected area was painless on palpation. The skin on the lesion site was free of scabs and traces of hair growth were seen. The lesion site was swabbed and cultured. It was negative for *P. aeruginosa*. Administration of gentamicin for nine-day period, improved skin lesions (Fig. 2A). At the end of the ninth day, the affected area was painless on palpation and hair growth were seen. Daily administration of glycerin for 12 days had no effect on cutaneous *P. aeruginosa* induced lesions. At the end of the 12th day, the affected area was still swollen and painful. The centre of some of the swellings was

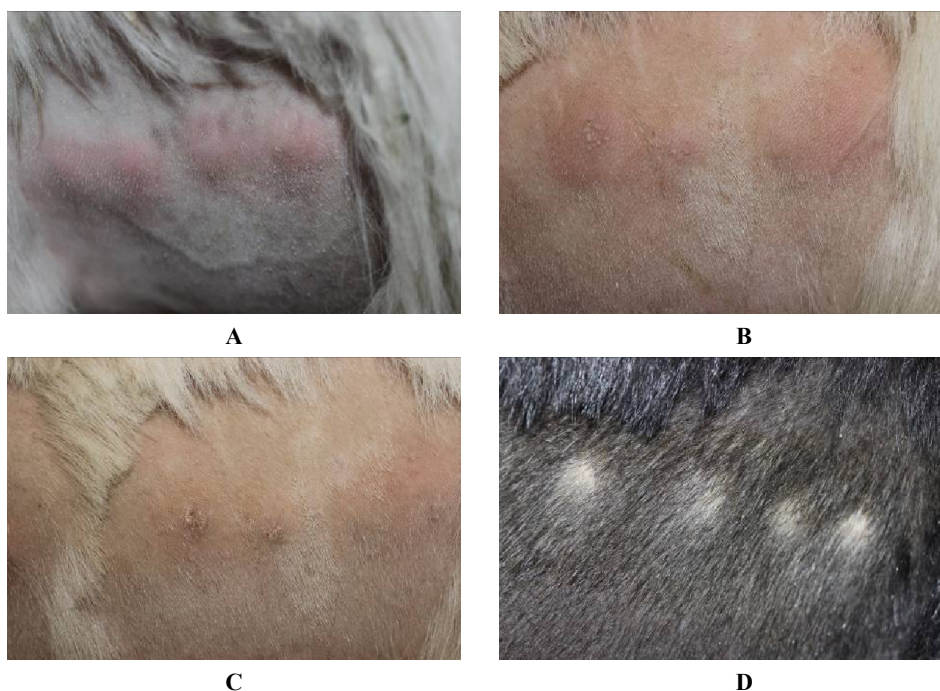


Fig. 1. **A.** Intradermal inoculation site of *P. aeruginosa* one day after inoculation. The site lesions are swelled and reddened; **B.** *P. aeruginosa* inoculation site four days after inoculation. Small vesicle-like clusters have formed on lesions areas; **C.** Site of intradermal inoculation of *P. aeruginosa*, five days after inoculation. Crust or scab is formed on the skin protrusions following the rupture of vesicles; **D.** *P. aeruginosa* inoculation site on the ninth day of 1,8-cineole treated group following daily administration: the skin lesion improved fully.

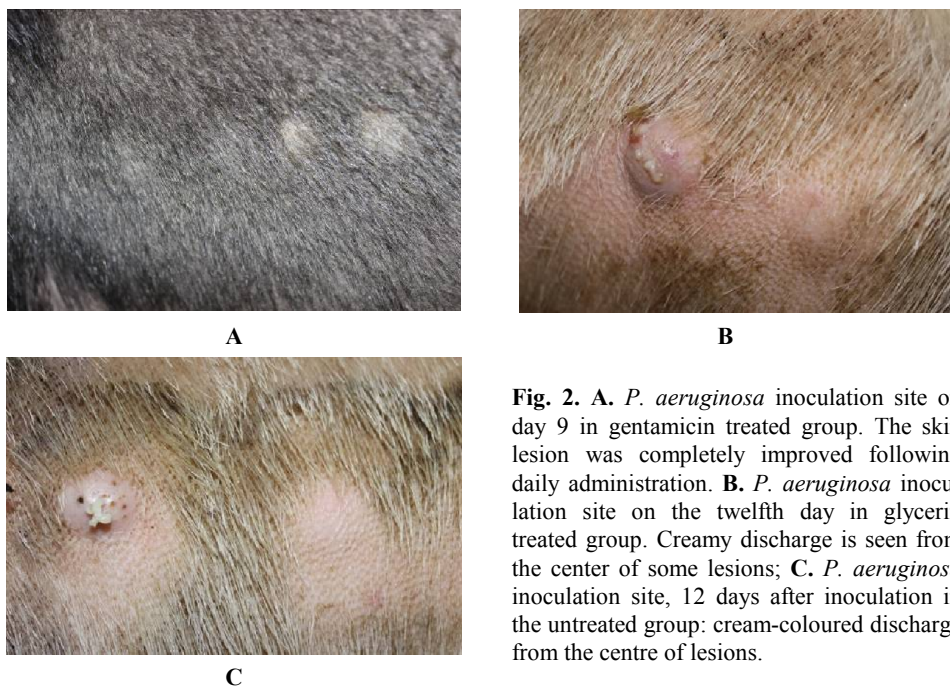


Fig. 2. **A.** *P. aeruginosa* inoculation site on day 9 in gentamicin treated group. The skin lesion was completely improved following daily administration. **B.** *P. aeruginosa* inoculation site on the twelfth day in glycerin treated group. Creamy discharge is seen from the center of some lesions; **C.** *P. aeruginosa* inoculation site, 12 days after inoculation in the untreated group: cream-coloured discharge from the centre of lesions.

slightly sunken and with creamy discharge (Fig. 2B).

Cutaneous lesions following intradermal inoculation of *P. aeruginosa* in the untreated group, similar to the glycerin-treated group, persisted and were painful until the 12th day. The centre of some of the swellings was slightly sunken and demonstrated creamy discharge (Fig. 2C).

DISCUSSION

The issue of antibiotic resistance is very serious today. One of the drug-resistant bacteria is *P. aeruginosa*. For this reason, research to obtain antimicrobials from other sources such as medicinal plants and their essential oils seems necessary. Use of plants such as eucalyptus (Eslami *et al.*, 2016), *Matricaria* genus (Sharifi-Rad *et al.*, 2018), *Ferula gummosa* (Bahmani *et al.*, 2016), garlic (Bahmani *et al.*, 2016),

Satureja khuzestanica and *Zataria multiflora* (Khaledi & Meskini, 2020), thyme (Burt, 2004) and green tea (Cox *et al.*, 2000) inhibited the growth of this bacterium *in vitro*.

In 1996, MIC of 10 mg/mL was reported for the methanolic extract of *E. globulus* in association with *P. aeruginosa* (Navarroa *et al.*, 1996). Similarly, Srinivasan *et al.* (2001) confirmed the growth inhibitory halo diameter of the plant extract for this bacterium: 32 mm and introduced it as a suitable disinfectant. Essential oils obtained from some members of the *Eucalyptus* genus contain 44.3–84.3% 1,8-cineole.

1,8-cineole is a terpene oxide compound found in the essential oils of many plants. Its inhibitory effects on the growth of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis*, and *Klebsiella* species have recently

been demonstrated. Gram-positive bacteria are more sensitive than Gram-negative bacteria to the inhibitory effect of plant derivatives. Also, the inhibitory effect on fungal organisms was confirmed, of which *Candida albicans* was the most sensitive. Some plant essential oils, such as *Eucalyptus* essential oil, contain 44.3–84.4% 1,8-cinnamol. In recent years, antimicrobial, antifungal, antimalarial, insecticidal, antiparasitic, antispasmodic and analgesic activity on the 1,8-cineole has been proven by scientific studies (Jalilzadeh-Amin & Maham, 2015). MICs of 1,8-cineole of 1,500, >2,000, 400, 1500, 300 and 400 $\mu\text{g.mL}^{-1}$ were reported for *Streptococcus mutans*, *Enterococcus faecalis*, *Streptococcus salivarius*, *Streptococcus sobrinus*, *Streptococcus mitis*, and *Streptococcus sanguinis* respectively (Bernardes *et al.*, 2010).

The inhibitory effect of 1,8-cineole on *P. fluorescens* ATCC 11253 (de Sousa *et al.*, 2012a,b) has been confirmed. However, 1,8-cineole has not been used clinically to treat cutaneous pseudomoniasis. On the other hand, many studies have shown that eucalyptol is safe when consumed in natural doses. The 1,8-cineole in *Eucalyptus* essential oil also has antioxidant and anti-inflammatory effects (Juergens *et al.*, 2003).

Compounds such as cineole, linalool, alpha-pinene, beta-pinene, borneol, limonene, carvacrol, gamma terpinene in the essential oils of most herbs have antifungal and antimicrobial properties (Hara-Kudo *et al.*, 2004). These compounds with high hydrophobic properties are able to isolate lipids from the bacterial cell wall, thereby increasing membrane permeability. Impaired bacterial cell membrane function leads to ion leakage and disturbance of membrane electron balance and lead to cell death (Cox *et al.*, 2000;

Duffy & Power, 2001; Lambert *et al.*, 2001; Carson *et al.*, 2002; Ultee *et al.*, 2002).

In the present study, 1,8-cineole administered for nine days improved the skin lesions caused by intradermal inoculation of *P. aeruginosa*. It seems that 1,8-cineole caused cell death and destruction of this organism by disrupting the permeability of *P. aeruginosa* membrane and disturbing its electronic balance, and also due to its anti-inflammatory activity (Juergens *et al.*, 2003), it improved skin lesions as quickly as possible.

It has been shown that *Mentha pulegium* L. contains high levels of cineole, which increased collagen synthesis and fibroblast activity following dermal use (Ehrnhöfer-Ressler *et al.*, 2013). It has also been shown that the use of essential oil containing cineole has significantly increased the growth of granulation tissue in the skin resection model, which may indicate fibroplasia (Ximenes *et al.*, 2013). Since fibroblasts are essential for collagen production, it is concluded that the use of ointments containing cineole can play an important role in increasing bud tissue by invoking fibroblasts to facilitate and accelerate the wound healing process (Khamaisi *et al.*, 2016; Mohammadi *et al.*, 2018). In future research, the therapeutic effects of 1,8-cineole in *Pseudomonas* spp. infections that occur naturally in animals can be discussed, as well as the mechanisms involved in inhibition of the growth of *P. aeruginosa* and skin repair promotion.

The results of this study showed that 1,8-cineole, similarly to gentamicin, healed goat cutaneous pseudomoniasis in nine days.

ACKNOWLEDGEMENTS

This work was funded as a dissertation of the first author, Farid Saeedi submitted as a partial fulfillment of the degree of Doctor of Veterinary Medicine (DVM) at Urmia University (Code 2017/1562).

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Paper received 30.06.2021; accepted for publication 24.10.2021

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