

In-vitro antibacterial activity of *Cymbopogon citratus* (DC.) Stapf. and *Eucalyptus globulus* Labill. essential oils against bacterial isolates from naturally mange mites infested goats

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Abstract

Background: Sarcoptic mange and associated secondary infections are challenges in improving productivity and production of goats in Ethiopia.

Objective: The present study has aimed at evaluating efficacy of essential oils from two plant species against opportunistic bacterial pathogens in mange infested goats.

Materials and methods: Essential oils (EOs) were obtained through hydrodistillation of freshly collected *Cymbopogon citratus* and *Eucalyptus globulus* leaves. Serial dilutions (20% to 0.3125%) of the EOs were tested for their antibacterial activity against *Staphylococcus aureus*, *S. hyicus* and *S. intermedium* using agar well diffusion and agar dilution methods and compared with standard antibiotics, erythromycin and chloramphenicol.

Results: *Cymbopogon citratus* and *E. globulus* EOs showed significant and dose dependent antibacterial activity. Essential oil of *C. citratus* was more potent than *E. globulus*; and *S. hyicus* was the most sensitive at 10% concentration. The EOs (10%) have comparable potency with the standard antibiotics. The minimum inhibitory concentration was found to be 0.125% v/v of *C. citratus* and 0.5% v/v of *E. globulus* EOs for all tested bacteria.

Conclusion: The two EOs showed strong antimicrobial property against the tested bacteria. However, further study is warranted to identify the specific potential EO combinations responsible for the effect, and formulation into appropriate dosage forms.

Keywords: antibacterial activity, essential oils, medicinal plants, opportunistic bacteria, sarcoptic mange

Introduction

Mange is a widespread and most important ectoparasitic disease of animals. Mange of goats is caused by four genera, namely; *Sarcoptes* sp., *Psoroptes* sp., *Chorioptes* sp. and *Demodex* sp. The *Sarcoptes* mange, *Sarcoptes scabiei* var. *caprae* in particular, is the most notorious ectoparasite of goats which is responsible for higher economic loss. Affected ewes produce less milk, lower production of live lamb per ewe and suboptimal growth rate (Soulsby 1982; Fthenakis et al. 2000; Fthenakis et al. 2001; Tadesse 2011). The feeding activity of *Sarcoptes* causes intense itching and scratching due to irritation, which causes self inflicted lesions and secondary bacterial infection that aggravate the conditions (Hay et al. 2012). The most common groups of etiologically important microorganisms involved in goat dermatitis are dermatophytic fungi, *Dermatophilus congolensis*, and ectoparasites. The bacteria, *S. aureus* and *S. hyicus* are also found as predominant dermatopathogenic species of microorganisms associated with skin lesions of goats (Mahanta et al. 1997). There is no scientific literature on bacterial species that are specifically associated

with mange mite infestation in goats. Whichever bacterial species is associated with mange mites infestations, broad spectrum conventional antibiotics have been in use to treat the secondary bacterial infections. However, due to the rapid development of multi drug resistant bacterial strains, which could mainly be emanated from irrational use of antibiotics and emergence of new mutant resistant strains, occurrence of bacterial infections that resist to be treated with conventional antimicrobial agents is increasing (Sieradski et al. 1999). Thus, it is vital to prospect development of new medicines including herbal medicines. Plant based antimicrobials represent a vast untapped source of medicines. Evidences show that they are effective in the treatment of infectious diseases in comparable degree with the conventional antimicrobials (Parekh et al. 2005). In Ethiopia, more than 800 plant species are recorded to be used in the traditional health care system (Geyid et al. 2003). Antimicrobial and wound healing plants are among some of the major medicinal plants that are commonly available in the Ethiopian markets (Dagne, 1996). Among the traditionally used medicinal plants, *C. citratus* (DC.) Stapf.

(Lemongrass) and *E. globulus* Labill. (blue gum) are used to treat different human and animal infectious diseases of parasitic, bacterial and viral origin elsewhere in Africa (Iwu 1999) and Ethiopia (Yineger et al. 2007; Lulekal et al. 2013; d'Avigdor et al. 2014). It is necessary to validate efficacy, safety and quality of the traditionally used medicinal plants so as to mainstream their formulation in the national and possibly international animal and/or human health care delivery system. Therefore, this study was aimed to evaluate the *in vitro* antibacterial activities of *C. citratus* and *E. globulus* against bacteria that cause secondary infection on mange infested animals.

Materials and methods

Plant material collection and extraction: Aerial parts of *C. citratus* and leaves of *E. globulus* were respectively collected from cultivation in Wondo Genet Agricultural Research Center (Ethiopian Institute of Agricultural Research) and around Kofele town of Oromiya region. Fresh leaves were then subjected to hydrodistillation to extract EOs. The extracts were filtered using Whatman No. 1 filter paper. The filtrates (essential oils) were used for their *in vitro* antimicrobial study.

Parasitological examination: Three naturally mange infested goats showing clinical signs like itching, dermatitis, intense pruritus, vesicles and macules appearance with thickened skin, scabs and loss hair were selected for sample collections (Radostitis et al. 2007). Infestation was confirmed through examination of skin scraping. The skin scraping was taken from multiple sites until blood capillary oozing was evident. The samples were then treated with 10% potassium hydroxide solution and placed on clean and dry glass slide. The samples were macerated with scalpel and covered with cover slip and examined under lower power microscope (Fthenakis et al. 2000). Identification of the species of the mites was performed according to the key morphological characteristics described by Wall and Shearer (2001). Goats with confirmed sarcoptic mange cases were used in the study for the isolation of bacteria responsible for secondary infection.

Microbiological assay: Samples were taken with sterile cotton swabs moistened with tryptose soya broth from the affected skin surface of the goat after disinfecting with 70% ethanol and removal of underlying debris to reduce the contaminant microorganism and then obtained the sample of exudates, vesicles or purulent discharge. Two swabs were rubbed firmly on the highly affected part of the skin and transferred into tryptose soya

broth, which was soon stored in a cool box maintained at 4 °C and later transported to the microbiology laboratory of Traditional and Modern Medicine Research Directorate, Ethiopian Public Health Institute (Quinn et al. 1994).

Bacteriological culture and isolation: The broth culture samples were aerobically incubated at 37 °C for overnight. The culture samples were thoroughly agitated and a loop of broth cultures were taken and streaked over labeled Petri-dishes containing blood agar base supplemented with 7% sheep blood (Quinn et al. 1994). The remaining samples in the test tubes were kept in a refrigerator at 4 °C until the end of the investigation. The growth of typical colonies on blood agar were characterized based on the presence or absence of hemolysis, the type of hemolysis and general appearance of the colonies including color, shape, size, consistency and other criteria after incubation at 37 °C for 24 h (Sharma and Adlakha, 1996). Single colony type from pure cultures on blood agar was transferred to nutrient agar for a series of primary and secondary biochemical tests (Carter 1994; Quinn et al. 1994). Isolates of *S. aureus*, *S. hyicus* and *S. intermedium* were then identified and used as test organism in the study. Preparation of bacteria for *in vitro* assay: Bacterial culture were prepared by sub culturing two to three colonies in 20 ml of nutrient broth and grown overnight at 37 °C. The turbidity of each cell suspension was adjusted with nutrient broth to match 0.5 MacFarland standards to 10⁸CFU/ml, which was then diluted to 10⁵-10⁴CFU/ml with one to ten dilutions and used for the experiment for appropriate inoculum suspensions.

Determination of antimicrobial susceptibility test: Antibacterial activity and minimum inhibitory concentration (MIC) of the EOs were respectively determined using agar-well diffusion and agar dilution methods. Agar-well diffusion standard method was adopted from Das et al. (2010). Standardized concentrations of inoculums were seeded on a sterile solidified Muller Hinton agar media. Then, ten mm diameter holes were punched aseptically on the seeded agar media with a sterile cork borer. A fixed volume (100 µL) of serially diluted EOs [ranging from 1% to 0.03125 % in 2% Tween-80 (v/v)] and the negative control (2% Tween-80) were then added to the respective wells. The plates were left for 1 h at room temperature to allow diffusion of the bioactive compounds of the crude EOs in the seeded agar media and subsequently incubated for 24 h at 35±2 °C. After incubation, zone diameter was measured to the nearest whole millimeter. The standard antibiotic discs of erythromycin and chloramphenicol (30µg

each) were used as positive control for the antibacterial activity test.

The MIC values of the EOs were determined by agar dilution method (Das et al. 2010). In brief, twofold serial dilution of each EO in a medium was prepared by adding two milliliter of the stock solutions that were prepared in 2% Tween-80 to 18 ml of agar so as to obtain the desired concentrations ranging from 1% to 0.03125 % (v/v). The prepared concentrations were then vortexed and poured into 90mm Petri-dishes. The agar was permitted to solidify in the plates on a level surface at room temperature. Negative controls containing only 2% Tween-80 were run simultaneously. The agar surface of the plates containing the dilution of EOs and the negative control were inoculated with 2µl aliquots of culture containing about 10^5 - 10^4 CFU/ml of each organism. The plates containing the lowest concentration of EOs were seeded first, and the negative control plates were seeded last to insure that viable organisms were present throughout the experiment. The inoculated plates were incubated at $35 \pm 2^\circ \text{C}$ for 24 h before being read. End-points for each EO were best determined by placing plates on a dark background and observing for the lowest concentration that inhibits visible growth, which is

recorded as MIC (expressed in micro liter per milliliter).

Statistical analyses: Descriptive statistical analyses were conducted and results expressed as $\pm$ SD of three independent experiments. One-way analysis of variance was also used to analyze significance difference in antimicrobial activity among different concentrations of the essential oils and standard drugs. Multiple comparisons were made among the experimental results using least significance test at 5% level of significance.

Results

The zones of inhibition that depict antibacterial activities of *C. citratus* and *E. globulus* EOs after 24 h incubation are respectively presented in Tables 1 and 2. The oils exhibited antibacterial activity against *S. aureus*, *S. hyicus* and *S. intermedium* that were included in the present investigations. Of the two plants included in this study, *C. citratus* showed higher antibacterial activity against all the tested bacteria than *E. globulus*. Furthermore, *S. hyicus* was the most sensitive bacteria than *S. aureus* and *S. intermedium* to the oils.

Table 1: Antibacterial activity of *Cymbopogon citratus* against clinical isolates of bacteria from mange mite infested goats

Essential oil	Concentration (%)	Diameter of inhibition zone (mm)		
		<i>S. aureus</i>	<i>S. intermedium</i>	<i>S. hyicus</i>
<i>C. citratus</i>	20	29.8 ± 0.5^a	30.0 ± 0.0^a	39.3 ± 1.0^a
	10	27.0 ± 0.8^b	27.3 ± 0.5^c	35.8 ± 1.0^b
	5	22.8 ± 1.0^c	24.8 ± 0.5^d	29.5 ± 0.6^c
	2.5	18.5 ± 1.2^d	19.3 ± 0.5^e	24.8 ± 1.5^d
	1.25	16.8 ± 1.0^e	17.8 ± 1.0^f	20.8 ± 0.5^e
	0.625	14.3 ± 0.5^f	13.0 ± 0.0^g	16.0 ± 0.5^f
	0.3125	12.3 ± 0.5^g	11.0 ± 0.0^h	13.0 ± 0.8^g
	Erythromycin (PC)	26.0 ± 0.0^b	29.0 ± 0.0^b	30.0 ± 0.0^c
Chloramphenicol (PC)	30µg disk	26.0 ± 0.0^b	27.0 ± 0.0^c	29.0 ± 0.0^c
Tween-80 (NC)	2% V/V	0.0^h	0.0^i	0.0^h

Values are means of three independent analyses $\pm$ SD and those that do not share the same letter within a column are significantly different using least significant difference test ($P \leq 0.05$)

Table 2: Antibacterial activity of *Eucalyptus globulus* against clinical isolates of bacteria from mange mite infested goats

Essential oil	Concentration (%)	Diameter of inhibition zone (mm)		
		<i>S. aureus</i>	<i>S. intermedium</i>	<i>S. hyicus</i>
<i>E. globulus</i>	20	24.3 ± 0.5^a	25.0 ± 0.0^b	25.3 ± 1.0^b
	10	20.3 ± 0.5^b	20.3 ± 0.5^c	21.0 ± 0.8^c
	5	17.5 ± 0.7^c	18.8 ± 0.5^d	17.5 ± 0.6^d
	2.5	15.3 ± 0.5^d	16.0 ± 0.8^e	15.0 ± 0.8^e
	1.25	11.8 ± 0.5^e	12.8 ± 0.5^f	12.8 ± 0.5^f
	0.625	0.0^f	0.0^g	0.0^g
	0.3125	0.0^f	0.0^g	0.0^g
	Erythromycin	26.0 ± 0.0^a	29.00 ± 0.00^a	30.00 ± 0.00^a
Chloramphenicol	30µg disk	26.0 ± 0.0^a	27.00 ± 0.00^a	29.00 ± 0.00^a
Tween-80	2% V/V	0.0^f	0.0^g	0.0^g

Values are means of three independent analyses $\pm$ SD and those that do not share the same letter within a column are significantly different using least significant difference test ($P \leq 0.05$)

Generally, the respective oils showed significant ($p < 0.05$) dose dependent growth inhibition on each test bacteria. The zones of inhibitions of the different concentrations of *C. citratus* EO were however higher

than parallel concentrations of *E. globulus*. Using agar well diffusion method, 10% and above concentrations of *C. citratus* oils showed equivalent if not significantly higher ($p < 0.05$) zone of inhibition as compared with the

standard drugs (30 µg disks of erythromycin and chloramphenicol) on *S. aureus* and *S. intermedium*. However, lesser concentration (5%) was adequate to inhibit growth of *S. hyicus* as standard drugs. Although the most diluted concentrations (0.625% and 0.3125%) of *C. citratus* showed significantly higher antibacterial activity than the negative control (2% Tween-80) ($p < 0.05$), *E. globulus* oil of the same concentration did not show any growth inhibition on all tested bacteria. Only the 20% (highest concentration) *E. globulus* oil has growth inhibition potential on *S. aureus* as the standard drugs. However, significantly lower inhibition zone than the standard drugs was recorded by all the EO concentrations tested on the other bacterial species. The MICs obtained by the agar dilution method were 0.125% v/v for *C. citratus* EO and 0.5% v/v of *E. globulus* EO against all tested organisms.

Discussion

The study deals with the antibacterial activity of *C. citratus* and *E. globulus* crude EOs against bacterial clinical isolates from naturally mange mite infested goats. The finding showed variation in antimicrobial activity of the EOs against the test organisms, and sensitivity of the organisms to the oils. Essential oil of *C. citratus* was more efficacious than *E. globulus* on all the bacteria, which is congruent with results of Cuéllar and Yunus (2009) against standard bacteria and clinical fungal microorganisms. Both EOs exhibited dose dependent growth inhibition on *S. aureus*, *S. hyicus* and *S. intermedium* showing maximum activity at the highest used concentration (20%) of the EOs using agar diffusion method.

The results obtained using agar diffusion method on *S. aureus* are in agreement with previous findings, although the MIC value found in the current study (0.125 v/v) was higher than the previous report (0.02% v/v) (Ruseniova and Parvanov 2009). The difference may be attributed to variation in the experimental conditions (method, temperature, photoperiod, etc), dispersion of the oil and the tested organism. Furthermore, EO compositions of plants are known to vary according to genetic, environmental and geographical conditions (Ganjewala 2009).

Previous investigations also demonstrated that numerous EOs have antibacterial and antimycotic properties. Similar investigations on the effect of *C. citratus* oils against bacterial species that induce human infection have also shown antimicrobial activity (Cuéllar and Yunus 2009; Bachir and Benali 2012; Nezhad et al. 2009). Its major EO, citral (both α -citral or geranial and β -citral or neral), exhibited strong antibacterial activity against *Haemophilus influenza*, *Streptococcus pyogenes*, *Streptococcus pneumonia* and *S. aureus* (Inouye et al. 2001), whereas myrcene (one of the EO components of *C. citratus*) is reported to have potentiating effect (Onawunmi et al. 1984).

Although the antimicrobial activity of *C. citratus* EO against human and animal pathogenic microorganisms is reported previously, no published information on the effect of the oil against *S. intermedium* and *S. hyicus*. The results of this study demonstrate that the EO has strong antibacterial activity against both *S. intermedium* and *S. hyicus*, whereby the later is more susceptible to all concentrations of the EOs. Although there is no statistically significant difference ($p > 0.05$) in efficacy among the concentrations and the positive controls, 20% and 10% *C. citratus* EO had better antibacterial activity than the positive controls (Erythromycin and Chloramphenicol), showing better potency of the EO at the specified concentrations against the test bacteria.

The present investigation has also revealed that *E. globulus* EO has antibacterial effect against *S. aureus*, *S. hyicus* and *S. intermedium* clinical isolates from mange infested goats. Although the antimicrobial activity of *E. globulus* EO has already been reported for various pathogenic microorganisms, no data has been obtained against *S. intermedium* and *S. hyicus*. The results of this study demonstrate that the EO has strong antibacterial activity against both *S. intermedium* and *S. hyicus*. *Staphylococcus hyicus* is more susceptible in all concentrations than the two tested bacteria. *Eucalyptus globulus* EO is known to contain about 73% 1, 8-cineole (1, 8- eucalyptol) and more than 46 other EOs (Song et al. 2009). The cumulative effect of many if not all of the EOs could be accountable to the antibacterial property of the crude EO. However, literature sources indicate that 1, 8- cineole, major and marker compound of *E. globulus*, is mainly responsible to the antimicrobial effect (Nezhad et al. 2009; Bachir and Benali 2012).

In the present study, stronger antibacterial property of *C. citratus* EO than *E. globulus* EO was observed in both agar diffusion and agar dilution methods. This finding is in agreement with previous investigation (Cuéllar and Yunus 2009). Previous report (Inouye et al. 2001) indicated that Citral (major EO of *C. citratus*) has stronger antibacterial effect than 1, 8-cineole (major EO of *E. globulus*). Therefore, the strength in antibacterial activity of the respective species could mainly be attributed to similar reason.

Despite higher number of goats in Ethiopia, different factors including infection by infectious organisms have contributed to significant loss in their production and productivity. Invasion by sarcoptic mange is one of the major contributors to the loss (Ebrahim 2010; Tadesse 2011). Currently, two different conventional drugs are being used to treat the mange and its opportunistic microorganisms. This practice has added burden on agro-pastoral communities in lowland areas where mange is highly prevalent and the country at large in terms of monetary expenditure and side effect on human and environment.

Targeting both the parasite and opportunistic pathogens with less costly and safe herbal formulation is expected to bring a promising output. Addis and coworkers (2013) have ascertained the acaricidal effect of both *C. citratus* and *E. globulus* EOs on sarcoptic mange and other ectoparasites of small ruminants. In this regard, use of optimized formulation of the EOs, targeting both the ectoparasite and opportunistic bacteria, could be a better option than using different conventional drugs of different toxicity level that target each organism.

Conclusions

The study showed that EOs of *C. citratus* and *E. globulus* have strong antibacterial activity against *S. aureus*, *S. hyicus* and *S. intermedium* isolates from naturally mange infested goats. A dose dependent inhibition was observed on the tested microorganisms. The demonstration of strong growth inhibition against all tested bacteria would appear to justify the rational basis in use of the plants against infectious diseases by indigenous communities in Ethiopia and their future potential in the formulation of new remedies that can be widely used for the treatment of animal and human microbial infections. It can also be inferred that use of optimized organic formulations containing *C. citratus* and *E. globulus* EOs that have dual effect (against the parasite and opportunistic bacteria) could be a better strategy to treat sarcoptic mange infested animals than using two different drugs, which independently target the parasite and opportunistic bacteria.

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