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RESEARCH ARTICLE

In vitro and *in vivo* Evaluation of Antimicrobial Activities of Essential Oils Extracted from Some Indigenous Spices

Rasheeha Naveed, Iftikhar Hussain, M Shahid Mahmood and Masood Akhtar[1, 2]

Institute of Microbiology; [1]Department of Parasitology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan; [2]Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan
*Corresponding author: driftikharuaf@gmail.com

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ABSTRACT

The study was conducted to investigate the antimicrobial activity of some indigenous essential oils (EOs) extracted by hydrodistillation from spices such as *Cuminum cyminum, Amomum subulatum, Cinnamomum verum* and *Syzygium aromaticum* against *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Bacillus subtilis, Aspergillus flavus* and *Candida albicans* by performing the disc diffusion assay and minimum inhibitory concentration (MIC) *in-vitro*, and *in vivo* antibacterial effect of EOs was studied in rabbits infected with *S. aureus* and treatment was done at infected site immediately with EOs. Among all treated groups, *C. verum* EO treated group showed significant decrease in viable bacterial counts at infected site after 24h and 48h of infection to 7.4×10^6 and 7.6×10^5 cfu, respectively, from 4.3×10^7 cfu before treatment. *C. verum* EO was found to be the most effective against *S. aureus* and *C. albicans*, showing zone of inhibition diameters 34±2.0mm and 50.3±8.6mm, respectively. The EOs, applied at different concentrations, showed variable inhibitory effects to all the microorganisms tested and more effective than control, both *in-vitro* and *in-vivo*. The results of the present study screened out the antibacterial and antifungal potential of the EOs, however further dose dependent studies, both *in-vitro* and *in-vivo* are required to find out their maximum safe levels against bacteria and fungi.

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INTRODUCTION

The most common causative agent of primary skin infections in humans is *S. aureus*. It is endemic in human populations and regarded as opportunistic bacteria, as it causes infections in children and immunocompromised patients. Topical infections by *S. aureus* are clinically significant and cause many serious symptoms including skin lesions (Kugelberg *et al*., 2005). Many pathogenic bacteria which have been of important concern to public health such as *E. coli, S. aureus, Salmonella spp, Clostridium spp* and *Yersinia spp* have been reported for numerous cases of intestinal disorders, causing vomiting and diarrhea (Oroojalian *et al*., 2010; Shahzad *et al*., 2012). Some bacterial strains including Gram positive and Gram negative bacteria are also associated with human periodontal health and disease (Ali *et al*., 2009). Fungal infections have increased systemic infections caused by *Candida* spp and other opportunistic pathogenic fungi causing major threat to many immunocompromised patients (Kamble and Patil, 2008). Pathogenic and toxigenic fungi are mostly treated by synthetic fungicides but the results are not successful due to their residual nature and toxicity to mammals (Singh *et al*., 2010). Commercial antimicrobial drugs have been used for combating infectious diseases for past few decades but prolonged use of these antibiotics has resulted in development of resistance and other side effects which have discouraged their use (Fu *et al*., 2007; Abbas *et al*., 2011a).

Most recently, there have been increasing efforts in finding safer and alternative means to reduce microbial growth (Abbas *et al*., 2010, 2011b, 2011c, 2012a, 2012b; Singh *et al*., 2010; Oskay, 2011; Zaman *et al*., 2012). Natural antimicrobial agents lessen the need of antibiotics, control microbial growth, remove undesirable pathogens, and minimize antibiotic resistance by strengthening immunity in humans (Tajkarimi *et al*., 2010; Rakashanda *et al*., 2012).

Among natural alternatives to antimicrobial agents, EOs is appealing approach. In the past, the antimicrobial activities of herbal EOs have been scientifically proved by

many researchers (Burt, 2004; Fisher and Philips, 2006; Oroojalian *et al*., 2010) thus could act as potential alternates to antibiotics against infectious diseases (Dongmo *et al*., 2009; Yasmeen *et al*., 2012). EOs is aromatic and volatile oily liquids concentrated in particular part of plants in special cells and the EO components are active against a variety of microorganisms (Skrinjar and Nemet, 2009). Spices EOs are well known for their antimicrobial effects against a wide range of microbes such as *S. typhi, E. coli* and *S. aureus* (Ozcan and Erkmen, 2001; Burt, 2004). The majority of EOs has been recognized safe and are recommended for use in traditional medicine (Burt, 2004; Chanthaphon *et al*., 2008; Kambil and Patil, 2008).

Keeping in view the antimicrobial effects of EOs, the present study was planned to investigate the antibacterial and antifungal effects of EOs, extracted from the commonly available botanicals in Pakistan, against some bacteria and fungi of public health importance *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plant material: Dried seeds of *C. cyminum* (cumin) and *A. subulatum* (large cardamom), bark of *C. verum* (cinnamom) and buds of *S. aromaticum* (clove) were purchased from local market of Faisalabad. The spices were identified by the Department of Botany, University of Agriculture, Faisalabad. The plant material was used in powder form after grinding with electric grinder.

Extraction of essential oils: Essential oils were extracted following the procedure described by Natta *et al*. (2008) with minor modifications. Spices (1000g) were subjected to hydro-distillation using hydrodistillation unit and the oil was extracted for 6 hours. A clear brown colored oil layer was obtained on the top of the aqueous distillate which was separated by separating funnel. The oil recovered was dried over anhydrous sodium sulphate and stored in a sealed glass vials at 4ºC.

Antimicrobial effect *in vivo*

Animals and infection induction: The experiments were conducted in the Animal laboratory house of Institute of Microbiology, University of Agriculture, Faisalabad, on 6-7 months aged rabbits of either sex and weight between 1.5-2.5 kg, at 25- 30^{o}C with standard food and water *ad libitum*.

Establishment of skin infection with *S. aureus* ATCC (25923) was performed according to the procedure described by Al-Basal, (2009) with some modifications. A total of 36 rabbits were grouped into six groups each having six rabbits. Each rabbit was injected subcutaneously with 0.1ml of *S. aureus* ATCC (25923) at concentration 1×10^{8}cfu/rabbit into shaved flank. Simultaneously, Group I-IV were treated with subcutaneous injection at infected site with EOs (0.48mg) 0.1ml, group V with s/c injection of Gentamycin 5 mg/kg at infected site and group VI was kept as untreated control. Rabbits were monitored for development of skin lesions, abscesses or inflammatory reaction on infected region through 48h.

Bacterial counts: Three rabbits from each group were sacrificed after 24h and 48h of infection. After disinfecting with ethanol (70%), the infected skin area and underlying tissue were taken out and homogenized in sterile saline. Quantification of bacterial counts were done by diluting the sample to 1:10 on nutrient agar plates and incubated at 37^{o}C for 24h. Bacterial counts were described as number of *S. aureus* cfu/g of tissue (Al-Basal, 2009).

Antimicrobial effect *in vitro*

Test microorganisms: Bacterial and fungal isolates were obtained from the culture collections of the Institute of Microbiology, University of Agriculture, Faisalabad. The isolates used were *E. coli* (ATCC 25922), *S. typhi* (ATCC 24682), *S. aureus* (ATCC 25923), *B. subtilis* (ATCC 6633), *A. flavus* (ATCC 204304) and *C. albicans* (ATCC 10231). These bacterial and fungal isolates were subcultured on Nutrient agar (Merck, Germany) and Sabouraud's dextrose agar medium (SDA, Lab M, UK), at 37°C for 24h and at 28°C for 3 days respectively (Gupta *et al*., 2008).

Disc diffusion assay: Disc diffusion method was used to determine antibacterial activity of the all above mentioned plants EOs against tested bacteria. The inoculums suspension 0.1ml (1.5×10^{5}cfu/ml) of each bacterial strain was sub-cultured on the Mueller-Hinton agar (MHA, Liofilchem, Italy) by using sterile glass spreader. Sterile 6 mm diameter filter paper discs (Whatmans filter paper No.1) were aseptically placed on Mueller-Hinton agar surfaces and 20µl of 500 mg/ml of plants EOs, dissolved in sterile DMSO (Dimethyl Sulfoxide, MP Biomedicals, France), was immediately added to discs. The plates were incubated at 37°C for 24h. Streptomycin disc (10mg/ml) was used as positive control (Natta *et al.*, 2008).

The fungal isolates were sub-cultured on SDA plates at 28ºC for 3-4 days. Oils were screened for their antifungal activity against test fungi by disc diffusion method. Inoculum 0.1ml of (1×10^{6}cfu/ml) suspension of the *C. albicans* and spores of *A. flavus* (5×10^{6}spores/ml) were applied on the SDA plates. The 20µl of each plant's EO was immediately added to filter paper discs placed on SDA plates. The plates were incubated at 28ºC for 48h (Kamble and Patil, 2008). Fluconazole, (15mg/ml) (Sabulal *et al*., 2006) was used as positive control (Gupta *et al*., 2008).

Minimal inhibitory concentration (MIC): The MIC of EOs was determined by a micro broth dilution assay in sterile 96-well micro titration plates (Schelz *et al*., 2006). Fifty µl of Mueller-Hinton broth (MHB, Liofilchem, Italy) was added in each well of a micro titration plate. The 50 µl of EO was pipetted into the first well of the plate. Subsequently two-fold serial dilution was prepared with Mueller-Hinton broth. The inoculum suspension (50µl) of each strain was then added in each well and mixed with a micro-pipette. All micro titration plates against all bacteria were incubated at 37°C for 24h and all fungi were incubated at 25°C for 48h. After incubation, the wells were examined for growth of microorganisms and the MIC was determined.

Statistical analysis: All the bacterial and fungal experimental results were expressed as means±S.E. One sample t-test was used for comparing means. Differences were considered significant at the level $P<0.05$. Each experiment was performed in triplicate.

RESULTS

Antimicrobial effect *in vivo*: The antibacterial effects of hydrodistilled EOs of *C. cyminum*, *A. subulatum*, *C. verum* and *S. aromaticum* against *S. aureus* were investigated in rabbits as experimental model. The viable bacterial count from skin and underlying tissue from EOs treated group after 24h and 48h were significantly much lower than untreated group and antibiotic treated group. Among EOs group, *C. verum*, *S. aromaticum* and *A. subulatum* showed remarkable decline in bacterial count from 24h to 48h as compared to *C. cyminum* (Fig.1).

However it was observed that small abscesses and inflammatory signs appeared in skin after 24-48h of infection in untreated group of rabbits. All above findings have indicated that the clearance of *S. aureus* from the infected site of rabbits by EOs were significantly higher as compared to untreated group. Furthermore, it was observed that while treating subcutaneous staphylococcal infection at infected site in rabbits, EOs proved to be more effective as compared to standard antibiotic used.

Antimicrobial effect *in vitro*: Antimicrobial activities of EOs were evaluated against test microorganisms using the disc diffusion method. The EOs showed varying degree of antimicrobial effects against microorganisms. The volatile oils of *C. cyminum*, *A. subulatum*, *C. verum* and *S. aromaticum* were found to be effective against both groups of bacteria. However, for gram positive bacteria, the highest antibacterial activity was shown against *S. aureus* which was maximum by the EO of *C. verum* followed by EO of *S. aromaticum*, *C. cyminum* and *A. subulatum*. In case of gram negative bacteria tested, *E. coli* and *S. typhi*, the widest spectrum of antibacterial activity was exhibited by EO of *C. verum* followed by *S. aromaticum*, *C. cyminum* and *A. subulatum* against *E. coli* (Table 1).

The results of the antifungal activities indicated that volatile EOs was more effective against fungi as compared to bacteria. All EOs showed inhibitory activity against both tested fungi but *C. albicans* was found more susceptible as compared to *A. flavus* and among tested EOs, the EO of *C. verum* showed excellent inhibitory activity with the biggest zone of inhibition against *C. albicans* followed by *A. subulatum*, *S. aromaticum* and *C. cyminum* (Table 2). Results indicated that EOs showed much greater inhibitory activity as compared to positive controls. Among all bacterial strains, *E. coli* was the most sensitive and *S. typhi* was least sensitive to Streptomycin. Fungi tested were sensitive to Fluconazole.

The result of MIC of EOs showed that *C. verum* EO was most effective in inhibiting the growth of both bacteria and fungi. *S. aureus* and *C. albicans* were most sensitive to *C. verum* EO by showing the lowest MIC whereas *S. typhi* and *A. flavus* were least sensitive to all EOs (Fig. 2).

Table 1: Zone of inhibition of different EOs at different concentrations against bacteria

EOs	Concentration (mg/disc)	*E. coli*	*S. typhi*	*B. subtilis*	*S. aureus*
	10	9±0.5	9±0.5	16±0.5	8.6±0.3
C. cyminum	5	8.6±0.3	8.6±0.6	12.3±0.8	8.0
	2.5	8.3±0.3	8.6±0.6	9.6±0.8	7.3±0.3
	1.25	8±0.5	7.3±0.3	8.6±0.6	7.0
	10	10.3±0.8	10.6±0.3	9.6±0.6	8.6±0.3
A. subulatum	5	9.6±0.6	9.3±0.3	9±0.5	8±0.5
	2.5	8.3±0.6	8.0	8.0	8.0
	1.25	7.0	7.0	8.0	7.0
	10	31±5.7	13.0	31.6±0.8	4±2.0
C. verum	5	16.3±0.8	10.6±0.3	27.6±0.8	27.6±0.3
	2.5	10.3±0.3	9.3±0.3	20.3±0.8	2±1.1
	1.25	8.3±0.3	8.3±0.3	12.3±1.4	0±1.5
	10	20±0.5	25.3±0.3	18.3±0.3	18.0
S. aromaticum	5	18.3±0.3	15.3±2.1	14.6±0.8	11.0±0.5
	2.5	10.6±0.3	8.3±0.6	10±0.5	9±0.5
	1.25	8.3±0.3	7.0	7.6±0.3	NA
Streptomycin	10	19.6±1.2	10.6±1.7	12.3±1.3	18.3±2.0
DMSO		NA	NA	NA	NA

Diameter of zone of growth inhibition is presented as mean±SE in mm; NA: No activity

Table 2: Zone of inhibition of different EOs at different concentrations against fungi

EOs	Concentration (mg/disc)	*C. albicans*	*A. flavus*
	10	16.6±0.8	22.3±0.3
C. cyminum	5	14.3±0.3	11.6±0.3
	2.5	12.3±0.8	7.3±0.3
	1.25	8.0	7.0
	10	25±0.5	27.6±0.3
A. subulatum	5	22.3±1.4	15.3±0.3
	2.5	18.3±1.2	13.3±0.3
	1.25	17±1.1	10.6±0.3
	10	50.3±8.6	41±0.5
C. verum	5	35±8.5	24.3±0.6
	2.5	30.6±7.8	15.3±0.3
	1.25	24.3±7.5	13±0.5
S. aromaticum	10	25±0.5	51.6±0.8
	5	23±0.5	47±0.5
	2.5	19±0.5	31.3±0.3
	1.25	16±0.5	14.6±0.3
Fluconazole	15	17±0.5	19±0.5
DMSO		NA	NA

Diameter of zone of growth inhibition is presented as mean±SE in mm; NA: No activity

DISCUSSION

Majority of the primary skin infections in humans caused by *S. aureus* are cellulitis, trauma, wound related infections and bacteremia which may also lead to severe complications. Immunity to *S. aureus* is achieved by complement-mediated killing by neutrophils and cell mediated immunity also play an important role in pathogenesis of lesions (AL- Basal, 2009).

A new animal model of rabbits for superficial and subcutaneous skin infection was established by *S. aureus* infection. All the EOs showed significant antibacterial activity at infected site by declining the viable bacterial count after 24 and 48h of infection as compared to the antibiotic and infected untreated rabbits. No previous findings or reports have been published for the efficacy of EOs on the complete clearance of subcutaneous *S. aureus* infection *in vivo*. The potency of EOs to inhibit *S. aureus* growth *in vivo* was significantly high which supports the presence of biologically active antibacterial agents in the EOs.

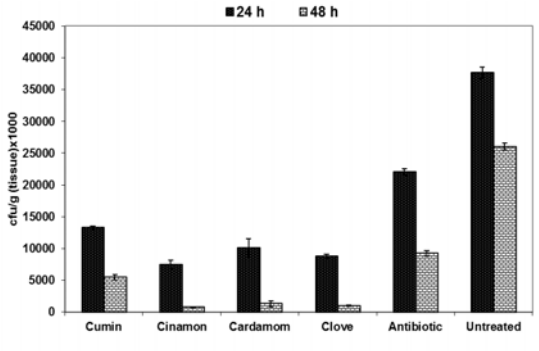

Fig. 1: Counts of *S. aureus* (mean±SE) at the infected site after 24h and 48h of infection and treatment.

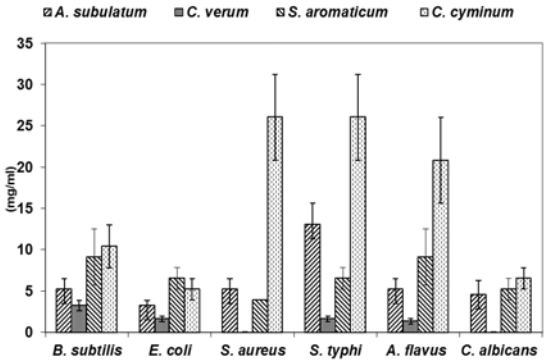

Fig. 2: Minimum inhibitory concentration (mean±SE) of essential oils against bacteria and fungi.

All EOs showed antibacterial and antifungal effects against all tested bacteria and fungi *in vitro*. However, there were variations in the extent of these antimicrobial effects because the antimicrobial activity depends on the type of spices, microorganisms as well as on the chemical composition and concentration of extracts and EOs (Skrinjar and Nemet, 2009).

The results showed the inhibitory effect of *A. subulatum* EO against all microorganisms however, Gram negative bacteria and fungi were identified as the highly sensitive. Like that of our findings, the EOs of another *Amomum* specie (*Amomum cannicarpum*) have also been found very effective against some gram negative bacteria and *Candida* species while gram positive bacteria were less sensitive. The main component present in *Amomum* oils is β-pinene which is responsible for the antimicrobial activity (Sabulal *et al*., 2006). Furthermore, *E. coli* and *C. albicans* were found sensitive to *A. subulatum* EO even at very low concentrations. Another study also showed that lower concentrations of *A. subulatum* EO are required to inhibit the growth of gram negative bacteria and *Candida* spp (Pattnaik *et al*., 2010).

C. verum EO also showed inhibitory effects against all microorganisms, however, *S. aureus* and *C. albicans* showed maximum sensitivity with low MIC. The antibacterial and fungicidal effect of EOs of some other species of *cinnamomum* (*Cinnamomum zeylanicum*) has already been reported against a wide range of bacteria and fungi (Gupta *et al*., 2008). The *cinnamomum* EO contains an aromatic aldehyde, cinnamonaldehyde, which is thought to give antimicrobial effect by inhibiting their growth. Another specie *Cinnamomum osmophloeum* EO has also been reported to have antibacterial effects (Chang *et al*., 2001).

Regarding the antimicrobial effect of *C. cyminum* EO, *B. subtilis* and *A. flavus* exhibited more sensitivity for this EO, however, *C. cyminum* EO was least effective among all tested EOs and only effective on higher concentrations. Some previous studies also reported the antimicrobial effects of *C. cyminum* EOs but these studies also concluded that only higher concentrations were effective. Generally, cumin aldehyde, γ-terpinene, p-cymene and β-pinene are mostly abundant components present in cumin oils account for biological activity (Jitrovetz *et al*., 2005). However, some studies have also shown the strong antifungal effect of *C. cyminum* EOs against *A. niger* and *Candida spp*. even at very low MIC (Kamble and Patil, 2008).

In present study, *S. aromaticum* EO exhibited inhibitory effect on all microorganisms of which *S. typhi* and *A. flavus* were highly sensitive. *S. aromaticum* EO showed lowest MIC against *S. aureus* and *C. albicans* indicating that these were highly susceptible for this plant. Similarly Fu *et al*. (2007) has also reported that low MIC of *S. aromaticum* EO was inhibitory against *S. aureus, E. coli, B. subtilis* and *C. albicans*. *S. aromaticum* EO is rich in eugenol which is well known for its antimicrobial activity against both bacteria and fungi (Oussalah *et al*., 2007). Moreover, these antimicrobial activities were comparable to commercialized antibacterial and antifungal drugs.

The difference in the antimicrobial activities of EOs between current results and the previous reports may be due to the different environmental growth conditions of botanical material and microbial *spp.* (Ozcan and Erkmen, 2001). Plant extracts and EOs may demonstrate different mechanism of action against bacterial strains as disturbance of cytoplasmic membrane resulting in increase permeability, loss of cellular material, damage of enzymes and destruction of genetic material (Kotzekidou *et al*., 2008). Comparison of published research with the current study is complicated as the result of a test depends upon the volume of inoculum, growth, culture and pH of media, incubation time and temperature.

Conclusion: In the face of drug resistance development, the EOs is the best alternative to the antimicrobial drugs. The results showed that the EOs of all five tested plants were effective against all bacteria and fungi. This effect was dose dependant, therefore, there is need to conduct experiments on large scale, both *in vitro* and *in vivo*, to establish the maximum safe levels of these EOs against studied bacteria and fungi. Moreover, histological examination of the infected and treated skin and tissues should be investigated to check the effect of the EOs on the inflammatory process.

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