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Antimicrobial Activity of Natural Respitol-B and its Main Components againsT Poultry Microorganisms

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Abstract: Poultries are infected to different kinds of microbial infections during their growth. For prevent of these diseases, many farmers use the synthetic antimicrobial agents. Whereas, the poultries participate in food cycle of human, the residues of these agents enter in human and cause many undesired side effects. In this study, the antimicrobial activity of Respitol-B and its main components (eucalyptus oil and menthol) was evaluated on different kinds of microorganisms including gram positive, gram negative bacteria, yeast and fungi *in vitro* conditions by disc diffusion and micro broth dilution assays. The gram positive bacteria, yeast and fungi is more sensitive than Gram negative ones to Respitol-B. *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium* is less sensitive to Respitol-B. Evaluation of menthol and eucalyptus oil for their antimicrobial activities exhibited that the antimicrobial activity of menthol is higher than that of eucalyptus oil. Eucalyptus oil had the best effect on *Vibrio cholerae*, *Staphylococcus aureus*, *Aspergillus flavus* but had no effect on others. The antimicrobial activity of menthol is observable and its presence in Respitol-B enhances the antimicrobial activity of Respitol-B. Respitol-B as a 100% herbal drug has antimicrobial effect and can be used as alternative therapy for preventing and controlling of infections.

Key words: Respitol-B, antimicrobial activity, eucalyptus oil, menthol, poultry microorganisms

INTRODUCTION

Microbial infections refer to the many and various diseases resulting from infection with different bacteria and fungi. In recent years, microbial infections are recognized as the major cause of morbidity and mortality in chickens and turkeys. These infections have spread rapidly among turkeys. Microbial infections may cause as primary infection or in combination with other microbial agents as secondary infections.

Plant products have received a renewed interest in their use as alternative source for various medical implications such as antimicrobial, anti-inflammatory, analgesic and antipyretic effects. Since early times, essential oils have been used as flavor and fragrance in perfumery, pharmaceutical, cosmetic and food industries and as antiseptic or therapeutic ingredients in folk medicine and aromatherapy. These essential oils also have different biological activities such as antibacterial, antiviral, antifungal effects and some of them can stimulate the responses of immunity system. With regard to these biological activity, a new product (Respitol-B) was made in two different pharmaceutical kinds (drinking and spraying form) for control and treatment of respiratory tract infections such as bronchitis and poultry influenza.

Respitol-B and its main components, eucalyptus oil (*Eucalyptus globulus*) and menthol is prescribed in severe and dry temperature especially in dryness of mucosal membranes of respiratory airways in chickens or when the application of antibiotics is not possible or the antibiotic resistance is developed.

Eucalyptus oil has been placed under GRAS (Generally Regarded as Safe) category by Food and Drug Authority of USA and classified as non-toxic oil (U.S. EPA, 1993). 1,8-cineole is the major component of different compounds in eucalyptus oil. In fact, 1,8-cineol is a typical compound in eucalyptus oil and is responsible for different pharmacological effects of eucalyptus oil (Duke, 2004). The antibacterial, antifungal, antiseptic,

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pesticide (Cimanga *et al*., 2002) and nematicidal (Ibrahim *et al*., 2006) effect of eucalyptus oil has been identified for hundreds of years.

Menthol is made synthetically or extracted from mint oils (Galeottia *et al*., 2002). The aim of this study was to evaluate the antimicrobial activity of Respitol-B and its main components (eucalyptus oil and menthol) on different kinds of microorganisms including gram positive, gram negative bacteria, yeast and fungi *in vitro* conditions.

MATERIALS AND METHODS

Chemical materials and their analysis: Respitol-B (Barij Essence Pharmaceutical Company, Kashan, Iran), menthol solution and a solution containing eucalyptus oil were prepared from Biology Center of Barij Essence, Kashan, Iran.

The chemical reagents (Respitol-B, menthol and eucalyptus oil) were analyzed by gas chromatography (GC) using a Varian 3800 apparatus with column of CP-Sil 8 CB (60 m×0.32 mm i.d., film thickness, 0.25 μm) equipped with a FID detector. The oven temperature programmed as follows: 50-230°C at 3°C min^{-1}, then held for 10 min at 230°C. Nitrogen was used as carrier gas at a flow rate of 7 psi. The injector and detector temperatures were 230 and 250°C, respectively. Components of volatile oil were identified by Retention Indices (RI) relative to standard components (Adams, 2001).

Microbial strains: In this study, we used the bacteria including: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6051, *Bacillus cereus* ATCC 1247, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* ATCC 1187, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 8739, clinical isolate of *Vibrio cholerae*, field isolate of *Aspergillus flavus*, *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231.

Disc diffusion method: The agar disc diffusion method was employed for the determination of antimicrobial activity of compounds. Briefly, using a sterile cotton swab, 0.1 mL from 10^8 and 10^6 CFU mL^{-1} microbial suspensions were spread on the Mueller Hinton Agar and Sabouraud dextrose Agar for bacteria and fungi, respectively. Sterile filter paper discs (6 mm in diameter) were impregnated with 20 μL of the each solution and were placed on the inoculated plates. These plates, after remaining at 4°C for 2 h, were incubated for 24 h at 37°C (for bacteria) and 48 h at 30°C (for fungi). The diameters of the inhibition zones were measured in millimeters. All tests were performed in triplicate (Wikler, 2009).

Determination of minimum inhibitory (MIC) and lethal (MLC) concentrations: The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values of compounds were determined by micro broth dilution assay. The oil was twofold serially diluted with 10% DMSO which contains 1000-15.625 μg mL^{-1} of each component. These dilutions were prepared in a 96-well micro titre plate. MOPS-buffered RPMI 1640 (for fungi), cation adjusted Muller Hinton broth (Marchetti *et al*., 2000) was used as broth media. After shaking, 100 μL of each component was added to each well. The above microbial suspensions was diluted (1×10^6 CFU mL^{-1} for bacteria; 10^4 for fungi) and then 100 μL was added to each well and incubated at 35±2°C. MICs were defined as the lowest concentration of compound that inhibits bacteria after 24 and fungi after 48 h. MLC values were the first well that showing no growth on solid media.

Statistical analysis: All experiments were performed in triplicate. The means of each experiment were determined by SPSS software (version 17, Chicago, Illinois, USA).

RESULTS AND DISCUSSION

The analysis of respitol-B exhibited the presence of 4 major components that represent 97.52% of total product's composition. There are including menthol (50.7% or 100 mg mL^{-1}), 1,8-cineol (41.5% or 82 mg mL^{-1}), limonene (3.5%) and α-pinene (1.9%) (Table 1).

The antimicrobial activity of Respitol-B and its main components and their potency were assessed by determining the Inhibition Zone diameter (IZ) and MIC and MLC as given in Table 2.

Table 1: Chemical composition of respitol-B, menthol, eucalyptus oil

Components	Repitol-B (%)	Menthol (%)	Eucalyptus oil (%)	RI
α-pinene	1.9	-	3.7	932
limonene	3.5	-	12.7	1024
1,8-cineole	41.5	-	78.2	1026
menthol	50.7	98	-	1167

RI: Retention indices

Table 2: Antimicrobial activity of respitol-B and main components

	Respitol-B			Menthol			Eucalyptus oil		
	IZ	MIC	MLC	IZ	MIC	MLC	IZ	MIC	MLC
S. aureus	16.4	125	250	22.8	125	250	14.2	62.5	250
B. cereus	13.4	250	>1000	23.4	250	>1000	0	250	>1000
B. subtilis	12.0	250	>1000	21.7	125	>1000	6.8	250	>1000
E. coli	NE	250	500	8.8	250	500	NE	250	500
P. aeruginosa	NE	250	500	NE	250	250	NE	250	500
S. marcescens	9.2	250	500	12.7	250	250	7.5	250	500
K. pneumoniae	43.3	250	250	>70	125	125	8	250	250
S. typhimurium	8.4	250	500	9.6	250	250	8.2	250	500
V. cholerae	19.1	250	1000	24.1	250	500	14.7	250	1000
C. albicans	25.2	125	500	39.5	125	500	6.5	125	500
A. niger	14.8	125	500	35.0	125	500	8.0	125	500
A. flavns	23.0	125	250	30.0	125	250	12.5	125	250

IZ: Inhibition zone diameter (mm), MIC: Minimal inhibitory concentration (μg mL^{-1}), MLC: Minimal lethal concentration (μg mL^{-1}), NE: No Effect

The gram positive bacteria, yeast and fungi were more sensitive than gram negative ones to Respitol-B. *P. aeruginosa*, *E. coli* and *S. typhimurium* were less sensitive to Respitol-B. Evaluation of menthol and eucalyptus oil as the main components of Respitol-B for antimicrobial activity exhibited that the antimicrobial activity of menthol was higher than that of eucalyptus oil. Eucalyptus oil had the best effect on *V. cholerae*, *S. aureus*, *A. flavus* but had no effect on others. Menthol had good antimicrobial effects against gram positive bacteria and fungi, but the gram negative bacteria were less sensitive to menthol. Menthol exhibited high antifungal activity against *A. flavus*, *A. niger* and *C. albicans*.

Eucalyptus is common ingredient in over the counter cold remedies today and long used as an effective treatment for colds, flu, sore throats, bronchitis and pneumonia. Eucalyptus is excreted from the body through the lungs and urine, so, it is useful for upper respiratory and urinary tract infections. Its leaves have also been used as an effective flea repellant. *E. globulus* exhibited the antimicrobial activity against *E. coli* $O_{157}H_7$ (Moreira *et al*., 2005), *Trichophyton mentagrophytes* (Takahashi *et al*., 2004), gram positive, gram negative bacteria and fungi (Pattnaik *et al*., 1996). Also, camphor and 1,8-cineole, the main components of eucalyptus oil are reported to have antimicrobial properties (Hammerschmidt *et al*., 1993; Carson and Riley, 1995). Camphor and 1,8-cineole-supplemented feed in compared to un supplemented feed (Allen *et al*., 1997) such as respitol-B containing 1,8-cineole and camphor can help to weight gain when turkeys or chickens infect with bacterial or fungal microorganisms. At first, the antimicrobial activity of 1,8-cineole in short time was higher than that of camphor but after 60 min, this activity gradually decreased and the antimicrobial activity of camphor was higher than that of 1,8-cineole. 1,8-cineole is a lipophilic compound with greater affinity to cell membranes but camphor has less lipophilic property with better activity on *S. aureus* (Mahboubi and Kazempour, 2009). Its antiviral activity (Cermelli *et al*., 2008) and expectorant properties (Leung and Foster, 1996) of eucalyptus oil is reported. This study showed the antimicrobial activity of eucalyptus oil is compatible with other studies, but this effect is less than that of menthol.

Menthol ($C_{10}H_{20}O$) a waxy, crystalline substance, clear or white in color is a terpenoid from mint family (*Mentha* spp), such as peppermint.

Bupesh *et al*. (2007) showed that menthol has fungicidal against *C. albicans*, *A. albus* and dermatophytic fungi. The best antimicrobial activity of menthol against gram positive bacteria *S. aureus* than the gram-negative *E. coli* was reported, also menthol migrate good in aqueous medium and interact with phospholipic membranes (Trombetta *et al*., 2005). The antimicrobial activity of menthol is observable and its presence in respitol-B enhances the antimicrobial activity of Respitol-B. The gram negative bacterium *P. aeruginosa* resisted to all compounds. (Al-Bayati, 2009) reported that *P. aeruginosa* is only microorganisms that is resistance to menthol in all concentrations also menthol had good antifungal activity against *C. albicans*. *P. aeruginosa* is a highly relevant opportunistic pathogen. One of the most characteristics of *P. aeruginosa* is its low antibiotic susceptibility. This organism causes diseases as an opportunistic organism and may occur through skin wounds, contaminated vaccines or antibiotic solutions and so on in poultry. Respitol-B has less activity against this bacterium *in vitro* condition but it is a good natural compound that is effective against different kinds of microorganisms especially gram positive bacteria, yeast and fungi.

CONCLUSION

In recent years, microbial infections have become recognized as a major cause of morbidity, mortality and condemnations in chickens and turkeys. In conclusion, the Respitol-B was able to reduce the population of different microorganisms and has diverse biological activity such as opening and disinfecting of respiratory airways, secretion of mucosa, anti-inflammatory and topical analgesic. Respitol-B is a natural product comprised mainly of essential oil which has been proven safe for chickens; also the efficacy of this product in preventing respiratory problems, improving performance and stimulating the immune system in drinking water has been demonstrated. Clinical trial of respitol-B has to be performed in field against infectious diseases that is caused by microorganisms and compared to conventional treatment for such diseases.

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