Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
18 (Supl.): 670-675, Dez. 2008
Received 10 October 2008; Accepted 16 November 2008

Artigo

In vitro anti-staphylococcal activity of *Hyptis martiusii* Benth against methicillin-resistant *Staphylococcus aureus*-MRSA strains

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RESUMO: "Atividade anti-estafilocócica *in vitro* de *Hyptis martiusii* Benth contra linhagens de *Staphylococcus aureus* resistentes à meticilina - MRSA". Este é o primeiro relato de atividade antibacteriana de *Hyptis martiusii* Benth. Neste estudo, o extrato etanólico de *H. martiusii* foi avaliado para atividade antimicrobiana contra linhagens de *Escherichia coli*, *Pseudomonas aeruginosa* e *Staphylococcus aureus*. O crescimento de todas as bactérias testadas foi inibido pelo extrato. O diâmetro das zonas de inibição variaram de 13 - 20 mm. Os valores da CIM e CBM variaram de 128 a ≥ 1024 mg/mL e 256 a ≥ 1024 mg/mL, respectivamente. Devido a isso, podemos indicar que o extrato etanólico de *H. martiusii* pode ser usado como um agente anti-*Staphylococcus*. Quando comparado com outros antibióticos como meticilina e gentamicina, o extrato foi mais efetivo, demonstrando ser um promissor agente antibacteriano.

Unitermos: *Hyptis martiusii*, Labiatae, atividade anti-estafilocócica, atividade antimicrobiana.

ABSTRACT: This is the first report about the antibacterial activity of *Hyptis martiusii* Benth. In this study the ethanol extract of *H. martiusii* was tested for its antimicrobial activity against strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The growth of all bacterial strains tested was inhibited by the extract. The diameter of inhibition zones varied from 13 to 20 mm for the extract. The MIC and MBC values ranged from 128 to ≥ 1024mg/mL and 256 to ≥ 1024 mg/mL, respectively. It is therefore suggested that extracts from *H. martiusii* could be used as an anti-*Staphylococcus* agent. Compared with methicillin and gentamicin, the extract was more effective, being a promising antibacterial agent.

Keywords: *Hyptis martiusii*, Labiatae, anti-staphylococcal activity, antimicrobial activity.

INTRODUCTION

Staphylococcus genus is widely spread in nature being part of the indigenous microbiota of skin and mucosa of animal and birds. Some *Staphylococcus* species are frequently recognized as etiological agents of many animal and human opportunistic infections (Nostro et al., 2004). *S. aureus*, *S. epidermidis*, *S. saprophyticus a*nd *S. haemolyticus* are the most important species as community and nosocomial human infection causing agents. In addition of causing different kinds of intoxications, *S. aureus* has been the most common etiological agent of festering infections that attack different tissues and/or organs (e.g. furuncle, carbuncle, abscess, myocarditis, endocarditis, pneumonia, meningitis, bacterial arthritis) (Verhoeff et al., 1999; Pereira et al., 2004). Capsule, peptidoglican, teicoic acids, adesins and synthesis of enzymes and extracelullar toxins are some virulence attributes present in/on *S. aureus* cell (Nostro et al., 2004).

Among the bacterial genera able to develop changes in their sensitivity to antimicrobials, *Staphylococcus* species have been recognized as having increasing and worrying antimicrobial resistance (Georgopapadakou, 2002; Nostro et al., 2004). For patients, the antimicrobial resistance increases the morbidity and mortality, whilst for healthcare institutions it means increasing costs (Dancer, 2001; Coutinho et al., 2005). Regarding the increasing clinical importance given to nosocomial and community bacterial infections and the progressive development of antimicrobial resistance, a great number of scientific researches emphasizing the antibacterial properties of plant products has been carried out (Hernández et al., 2003; Silva-Santos et al., 2004; Duarte et al., 2005; Gayoso et al., 2005; Michelin et al., 2005; Lima et al., 2006a,b;

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ISSN 0102-695X

Santos et al., 2007; Serafin et al., 2007; Silva et al., 2007; Aguiar et al., 2008; Silva et al., 2008; Salvagnini et al., 2008; Simões et al., 2008). Filtrates, infusions, macerated, juices, extracts and cataplasms from plants with medicinal properties have been applied in the treatment of various diseases since antiquity (Annuk et al., 1999; Hernández et al., 2003).

Hyptis martiusii Benth ("cidreira-do-campo") is a small shrub belonging to family Labiatae used in Brazilian traditional medicine against intestinal and stomachic diseases (Agra et al., 2008), with few pharmacological reports. Antitumoral, cytotoxic and insecticidal activities were identified (Araújo et al., 2003; Costa-Lotufo et al., 2004; Costa et al., 2005; Araújo et al., 2006), but no antimicrobial activity has so far been reported according to a literature survey.

Chemical compounds as flavonoids (Isobe et al., 2006), triterpenes (Falcão et al., 2003), diterpenes (Ohsaki et al., 2005) and sesquiterpenes (Facey et al., 2005), with antimicrobial, insecticidal, analgesic, antiplasmodial activities and antidepressive effect (Okiemy-Andissa et al., 2004; Fragoso-Serrano et al., 2005; Chukwujekwu et al., 2005; Isobe et al., 2006; Silva et al., 2006; Bueno et al., 2006) were isolated on other plants from the genus *Hyptis*.

This study was carried out with the purpose of evaluating the antimicrobial effect of the ethanolic extract of *H. martiusii* to inhibit the growth and survival of *S. aureus* strains isolated from clinical samples.

MATERIAL AND METHODS

Drugs

Methicillin (SIGMA), Gentamicin (SIGMA). The solutions of antibiotics were prepared using the recommendations of Clinical and Laboratory Standards Institute - CLSI (NCCLS, 2003).

Strains

Escherichia coli (ATCC 8539 and ATCC10536), *Pseudomonas aeruginosa* (ATCC 25619 and 9027), *Staphylococcus aureus* (ATCC 6538 and 25923) were used as positive control. The clinical and methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from the Laboratório de Genética de Microrganismos - UFPB. All strains were stocked at room temperature in heart infusion agar slants (HIA, Difco) and, prior to assay, the cells were grown overnight at 37ºC in brain heart infusion (BHI, Difco).

Plant material

Leaves of *Hyptis martiusii* were collected in the city of Crato, State of Ceará, Brazil. The plant material was identified by Dra. Maria Arlene Pessoa da Silva and voucher specimen have been deposited with the number 464 at Herbarium "Dárdano de Andrade Lima" of Universidade Regional do Cariri - URCA.

Preparation of ethanol extract from *Hyptis martiusii* (EEHM)

200 g of aerial parts were oven-dried at room temperature and powdered. The powdered material was extracted by maceration using 1l of 95% ethanol as solvent at room temperature. The mixture was reserved for 72 h at room temperature. The extracts were then filtered and concentrated under vacuum in rotatory evaporator (Brasileiro et al., 2006). For the tests, the extract was diluted in DMSO and its highest concentration remaining after dilution into broth caused no inhibition of bacterial growth.

Antimicrobial activity test

Solid medium diffusion technique using agar wells was used for screening the antibacterial activity. For this, 1 ml of the bacterium suspension (approximately 10^5 cfu/ml) was uniformly spread on sterile agar Muller-Hinton Petri dishes. 50 µl of EEHM 10mg/mL were addicted within agar wells with 6 mm diameter (modified from Nair et al., 2005; Sahin et al., 2004). The system was incubated at 37 °C for 24 hours. It was considered as positive antibacterial activity when observed growth inhibition zone with diameter ≥10 mm diameter (Lima et al., 1993). MICs were determined in a microtitre assay (Javadpour et al., 1996) by inoculation of 100 µL of each strain suspended in BHI two - fold concentrated (final concentration 10^5 colony-forming units/mL) in a 96-well microtitre tray with two-fold serial dilutions by adding 100 µL of EEHM solution. The final concentrations of the EEHM was 512, 256, 128, 64, 32, 16 and 8 µg/mL. The MICs were recorded as the lowest concentration for growth inhibition. The Minimal bactericidal concentration (MBC) was determined inoculating samples from non - growth wells on plates with BHI agar. The MRSA strains 007 and 441 were assayed with methicillin and gentamicin with final concentrations of 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 µg/mL. All plates were incubated aerobically for 24 h at 37 ºC. The MBCs were recorded as the lowest concentration without growth. All antimicrobial assays were performed twice and the results were expressed as average of the two repetitions.

Abbreviations

Oxacillin (OXA); Penicillin (PEN); inductive Erythromycin (EM^I); Kanamycin (KAN); Streptomycin (SM); Gentamicin (GEN); Amikacin (AMI); Tobramycin (TOB); Chloramphenicol (CHL); Tetracyclin-minocyclin (TCM); Neomycin (NEO); Paramomycin

(PARA); Butirosin (BUT); Sisomicin (SIS); Netilmicin (NET); Constitutive Erythromycin (ERI^C); Tetracyclin (TC); Ampicillin (AMP); Amoxicillin (AMOX); Cefalotin (CF); Cefadroxil (CFR); Cefalexin (CFL); Clindamycin (CN); Ciprofloxacin (CIP); Gatifloxacin (GAT); Ampicillin-Sulbactan (AMPS); Rifampicin (RIF); Novobiocin (NOV).

RESULTS AND DISCUSSION

In the last years there has been a great scientific interest in chemical and pharmacological investigations regarding the biological properties of medicinal plants (Almeida et al., 2001; Silva et al., 2003; Rocha et al., 2005; Barbosa-Filho et al., 2005; Barbosa-Filho et al., 2006a,b,c; Saúde-Guimarães & Faria, 2007; Barbosa-Filho et al., 2007; Biavatti et al., 2007; Oliveira et al., 2007; Barbosa-Filho et al., 2008). It is known that medicinal plants have been source of many drugs applied in clinical procedures (e.g morphine, emetine, rutine). The use of extracts as antimicrobial agents presents a low risk of rising microbial resistance to their action because are complex mixtures, making more difficult the microbial adaptability (Daferera et al., 2003).

Table 1 shows the inhibitory activity of EEHM against clinical isolates of *S. aureus*. EEHM showed effectiveness in inhibiting the all strains with inhibition zones between 13-20 mm (average: 16,5 ±1.8). Six strains showed inhibition zones with diameter ≥18 mm. Smallest inhibition zones (13 mm) were found on the MRSA strain 358, while the largest one (20 mm) were found on the MRSA strains 365, 10C and 19L. MIC and MBC values were 128 - 512 μg/mL and 256 ≥ 1024 μg/mL for the *S. aureus* strains, respectively. The effect was not observed on the *P aeruginosa* and *E. coli* strains. As far as we know, it is the first report of the antimicrobial activity of *H. martiusii*.

Table 2 shows the anti-staphylococcal efficacy of EEHM when compared with the aminoglycoside gentamicin and the β-lactam methicillin. The EEHM was 2 - 4 times more effective to inhibit the S. aureus growth than these drugs. Regarding the MIC values found for all assayed *S. aureus* strains, the classification criteria above cited confirms the strong anti-staphylococcal property of EEHM.

Plants remaining to the genus *Hyptis* are used in the folk medicine by populations around the world as an antimicrobial remedy (Goun et al., 2003; Wiart et al., 2004; Kala, 2005). These antimicrobial properties have been assayed and proven in ethanol extracts and essential oils of *H. ovalifolia* (Hasimoto and Souza et al., 2002; Souza et al., 2003), in methylene chloride extract from *H. brevipes* (Goun et al., 2003). Furthermore, flavones from *H. suaveolans* (Isobe et al., 2006) and pectinolides from *H. pectinata* (Fragoso-Serrano et al., 2005) showed antibacterial activity against *Helicobacter pylori*, *E. coli* and *S. aureus* respectively.

The results obtained in this study showed

Table 1. Origin, resistance profile of *Staphylococcus aureus* strains and inhibitory activity of *Hyptis martiusii*.

Strain	Origin	PRP[a]	Inhibition (mm)	MIC (μg/mL)	MBC (μg/mL)
E. coli ATCC10536	-	-	11	512	≥ 1024
E. coli ATCC8539	-	-	16	≥ 1024	≥ 1024
P. aeruginosa ATCC25619	-	-	16	≥ 1024	≥ 1024
P. aeruginosa ATCC9027	-	-	17	≥ 1024	≥ 1024
S. aureus ATCC6538	-	-	15	256	512
S. aureus ATCC25923	-	-	15	256	512
MRSA 06C	surgical wound	1	18	512	≥ 1024
MRSA 365	surgical wound	2	20	256	512
MRSA 358	surgical wound	2	13	128	256
MRSA 296I	abscess	3	14	512	≥ 1024
MRSA 171C	surgical wound	4	14	256	512
MRSA 015	surgical wound	2	14	512	≥ 1024
MRSA 02H	surgical wound	5	19	256	512
MRSA 01	windpipe secretion	6	14	512	≥ 1024
MRSA 007	surgical wound	2	19	256	512
MRSA 05H	surgical wound	7	17	128	256
MRSA 441	surgical wound	2	15	256	512
MRSA 10C	surgical wound	8	20	256	512
MRSA 192C	surgical wound	2	14	512	≥ 1024
MRSA 19L	surgical wound	9	20	256	512

A - Phenotipic Resistance Profile – PRP: 1 - Oxa, Pen, Em^I, Kan, Sm, Gen, Ami, Tob, Chl, Rif, Nov; 2 - Oxa, Gen, Tob, Ami, Kan, Neo, Para, But, Sis, Net; 3 - Oxa, Pen, Em^I, Kan, Sm, Gen, Ami, Tob, Rif; 4 - Oxa, Pen, Em^I, Kan, Sm, Gen, Ami, Tob, Tcm; 5 - Oxa, Pen, Em^I, Kan, Sm, Gen, Ami, Tob, Chl, Tcm; 6 - Oxa, Amp, Amox, Cf, Cfr, Cfl, Cn, Cip, Gen, Gat, Pen, Amps; 7 - Oxa, Pen, Eri^C, Kan, Sm, Gen, Ami, Tob, Tcm; 8 - Oxa, Pen, Eri^C, Kan, Sm, Gen, Ami, Chl, Tc; 9 - Oxa, Pen, Em^I, Kan, Sm, Gen, Ami, Tob, Chl,Tcm.

Table 2. Comparative effect of the ethanol extract of *Hyptis martiusii* (EEHM) and antibiotics against strains of *Staphylococcus aureus* isolated from clinical material.

Strain	MIC Methicillin (μg/mL)	MIC Gentamicin (μg/mL)	MIC EEHM (μg/mL)
MRSA 441	≥ 1024	≥ 1024	256
MRSA 007	≥ 1024	≥ 1024	512

the strong anti-staphylococcal property of *the ethanol extract of H. martiusii*, noted by small MIC value and effectiveness in inhibiting the microbial growth of *S. aureus*. These data are promising and could encourage further researches on phytochemical, toxicological and pharmacological aspects of *H. martiusii* by-products in order to support their possible rational use in the antimicrobial therapy, particularly, in anti-*S. aureus* therapy.

ACKNOWLEDGEMENTS

This work was supported by the following Brazilian agencies CNPQ and FAPESQ/PB.

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