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Ethanolic extracts obtained by extraction methods in the synthesis of *O. gratissimum* antibacterial agents

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The aim of this study was to define whether novel extraction methods such as microwave and ultrasound could obtain the most effective ethanolic extracts of *Ocimum gratissimum* as antibacterial agents. These extracts were compared with respect to extractive yield, eugenol content, antibacterial activity and brine-shrimp (*Artemia salina*) toxicity with extracts obtained by the classical procedures of maceration and Soxhlet. Significant differences among the extracts were observed in all analyses. Soxhlet extraction gave the highest yield (19.5%). Maceration and microwave extracts yielded the highest eugenol contents (11.6 and 11.8%, respectively). The bactericidal activity of the extracts was correlated with eugenol content (r_S = 0.894). Maceration gave the extract with the broadest spectrum of activity. Ultrasound methods yielded an efficient extract for use as a topical antiseptic (minimum inhibitory concentration (MIC) = 0.66 to 1.32 mg/ml for *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)). The most active extracts to treat vancomycin-resistant enterococci (VRE) infections were obtained by Soxhlet and microwave (MIC = 5.28 mg/ml). The extract obtained by maceration was the most toxic for brine shrimp, followed by the extracts obtained by ultrasonic horn, ultrasonic cleaning bath and Soxhlet /microwave. In conclusion, the antibacterial results showed that the extractive methodology can be chosen according to the intended use.

Key words: *Ocimum gratissimum*, microwave-assisted extraction (MAE), ultrasonic cleaning bath (UCB), ultrasonic horn (UH), antibacterial activity, toxicity, *Artemia salina*.

INTRODUCTION

With modern extraction methods such as ultrasound and microwave - assisted extraction , plant extracts can be obtained with higher yields and efficacy, less environmental impact and lower costs (Wang and Weller, 2006; Chen et al., 2007). Conventional extraction methods such as maceration and Soxhlet are based on

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the choice of solvent coupled with the use of heat and/or agitation, whereas ultrasound and microwave methodologies disrupt cell walls, improving mass transfer (Wang and Weller, 2006).

Ocimum gratissimum L. (Lamiaceae), known as tree basil, is widely used medicinally as a topical antiseptic and for the treatment of conjunctivitis, bronchitis and diarrhea, as well as a food flavoring (Onajobi, 1986; Silva et al., 2006). Antimicrobial activity was confirmed for the essential oil (Nakamura et al., 1999), ethanolic (Nweze and Eze, 2009; Passos et al., 2009), methanolic (Braga et al., 2007) and aqueous extracts (Junaid et al., 2006; Passos et al., 2009). This activity has also been correlated with a high content of eugenol (Nakamura et al., 1999).

To date, studies with *O. gratissimum* were performed only with extracts obtained by conventional methods (Junaid et al., 2006; Nweze and Eze, 2009; Passos et al., 2009). In some cases, extracts obtained with different plant materials (fresh or dried) or different solvents (water, hexane, methanol, ethanol or hydroalcoholic mixture) were compared (Junaid et al., 2006; Passos et al., 2009). The aim of this study was to define whether novel extraction methods such as microwave and ultra-sound could provide extracts of *O. gratissimum* that are more effective as antibacterial agents. Ethanolic extracts obtained by these methods were compared to those obtained with the classical procedures of maceration and Soxhlet , with respect to extractive yield, eugenol content, antibacterial activity, and toxicity to *Artemia salina*.

MATERIALS AND METHODS

Plant

Aerial parts of *O. gratissimum* L. used to obtain the ethanolic extracts were grown in Jardinópolis, São Paulo, Brazil. The plant material, previously identified by Dr Lin Chan Ming, was collected in March, 2007, dried in a ventilated oven at 45°C for three days and stored in closed dark packages until the extraction processes. To obtain eugenol from hydrolate, aerial parts identified by Dr Adelino Alvarez Filho were collected in December, 2006 and March, 2007 on the central Campus of the Universidade Federal de Santa Maria (UFSM), Santa Maria, Rio Grande do Sul, Brazil. Voucher specimens were deposited in the Biotechnology Department of the Universidade de Ribeirão Preto (no. 1329) and in the Herbarium of the Botany Department, UFSM (no. SMDB 11167).

Extraction and isolation of eugenol from hydrolate

Fresh aerial parts of *O. gratissimum* were steam-distilled for 3 h in a Clevenger-type apparatus (European Pharmacopoeia, 2007). After extraction, the hydrolate was collected and submitted to liquid-liquid and the residue was stored at -4°C in amber glass bottles until purification. The residue (210.7 mg) was submitted to a 15 g silica-gel chromatography column (CC, 1 × 29.5 cm) and eluted with dichloromethane at 1 ml/min, resulting in 14 fractions of 20 ml. Fractions were pooled according to their thin-layer chromatography (TLC) profiles and concentrated under reduced pressure at 40°C. Fractions 4 to 10 (103.8 mg) was analyzed by gas chromatography with mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). GC-MS analysis was performed on an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector, using an HP5-MS column (5% phenyl - 95% methylsiloxane, 30 m × 0.25 mm i.d. × 0.25 µm) and electron ionization-mass spectrometry (EI-MS) as detector (70 eV) according to Silva et al. (2010). NMR spectra were recorded on a Bruker DPX 400 FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$ with tetramethylsilane (TMS) as internal standard.

Ethanolic extractions of aerial parts

Dried aerial parts of *O. gratissimum* were extracted by maceration (MAC), Soxhlet (SOX), ultrasonic cleaning bath (UCB), ultrasonic horn (UH) and microwave-assisted extraction (MAE) with 95% ethanol in triplicate. The plant material was ground at 600 µm before extraction by the Soxhlet, ultrasonic and microwave-assisted processes. The extraction time, temperature and solvent volume/ plant material weight ratio (ml/g) of the different methods are presented in Table 1. Sonochemical procedures were carried out in a UCB (model Ultrasonic Cleaner 1440D - Series Evolution, 40 kHz and 100 W) and UH (model VC 750, Sonics, 20 kHz working frequency, 750 W). The horn was operated on a 40% cycle at 2.5 cm from the surface of the extraction solution. MAE was performed in a Multiwave 3000 microwave using a quartz closed-vessel system. The microwave energy program was up to 100°C, with ramp of 5 min until 300 W followed by 5 min of radiation. To finish, the vessels containing the extract were cooled for 30 min before opening. All extracts obtained were filtered, stored in closed amber bottles and concentrated under reduced pressure at 40°C. Afterwards, the extracts were placed in a desiccator and kept at room temperature until constant weight. Yields are expressed as a percentage of extract obtained from plant material (w/w).

Purification and quantification of eugenol in ethanolic extracts

The eugenol purification process from the ethanolic extracts of *O. gratissimum* was developed to reduce interference from other compounds with the quantitative analyses. Procedures were initially performed in a pilot CC and repeated for quantitative determination. Extracts (about 400 mg) were added to a CC (1 × 29.5 cm) containing 15 g of silica-gel 60 (Merck, 70-230 mesh) and eluted with chloroform-toluene 85:15 (v/v) at 2 ml/min. Fractions of 20 ml were obtained and monitored in pilot columns by TLC (silica gel F254, chloroform-toluene 85:15 v/v, detection: anisaldehyde-H_2SO_4) to detect the presence of eugenol. In the quantitative procedure, fractions that contained eugenol according to the pilot-column results (MAC: fractions 6-13; SOX: fractions 5-15; UH: fractions 5-14; UCB: fractions 5-12; MAE: fractions 3-13) were con-centrated under reduced pressure at 40°C. The resulting residue was diluted to 50 ml in a volumetric flask using the same eluent and the eugenol content was determined by gas chromatography with flame ionization detection (GC-FID). To determine the reprodu-cibility of the CC procedure, fractions without partition with hexane. The organic phase was evaporated at 40°C eugenol were compared by TLC with the corresponding fractions of the pilot

column. The eugenol was quantified by analyzing the area below the curve (N = 2). Eugenol previously obtained from the hydrolate of *O. gratissimum* (diluted in dichloromethane at 1.038 mg/ml) was used as external standard. GC-FID analysis was performed on a Varian gas chromatograph Model 3800 coupled to a flame ionization detector, using the Star Workstation 6.6 system for data acquisition and a CPSil 5CB column (100% methyl silicone, 30 m × 0.25 mm i. d. × 0.10 µm film thickness). Operating conditions: injection volume 1 µL; injector temperature 220°C; oven temperature program 40 to 260°C; 40°C for 4 min; ramp rate 4°C/min; detector temperature 310°C. The eugenol content was expressed as the percentage of eugenol in relation to extract (w/w).

Antibacterial activity

The antibacterial activity of the extracts was assayed by the broth microdilution method as established by M7-A6 (Clinical and Laboratory Standards Institute (NCCLS), 2003) for bacteria. The microorganisms tested included ATCC and clinical isolates from the University Hospital of Santa Maria (Table 2). Extracts were solubilized in 95% ethanol and serial dilutions were performed in culture medium to obtain concentrations of 10.550 to 0.020 mg/ml for *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 51299 and of 2.637 to 0.005 mg/ml for the other strains. Each extract was assayed three times, in triplicate. Ampicillin was used as the reference antimicrobial control according to the antimicrobial resistance profile. Negative (100 µl of Muller-Hinton broth), positive (100 µl of Muller-Hinton broth and 100 µl of inoculum) and product controls (180 µl of Muller-Hinton broth and 20 µl of stock solution) were also performed for each test. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the sample that prevented visible growth. Minimum bactericidal concentrations (MBCs) were defined as the lowest concentration yielding negative subcultures.

Brine-shrimp lethality test (BSL)

The BSL was carried out by using nauplii of brine shrimp *Artemia salina* (Leach), according to the methodology described by Silva et al. (2010). Extracts of *O. gratisssimum* (60 mg) were dissolved in 95% ethanol (1 ml) and diluted in artificial sea water to obtain final concentrations of 1000, 600, 300, and 150 µg/ml. Plates were maintained at 22 to 29°C for 24 h and the number of survivors was counted. Negative controls were tested with 95% ethanol diluted 1:100 in artificial sea water. All assays were performed in two repetitions (N = 3 per repetition).

Statistical analysis

Data are presented as the mean ± standard error of mean (SEM). To verify the homogeneity of variances, all data were submitted to a Levene test. Arcsine transformation was used for data on extractive yield and eugenol composition before statistical analysis. Results were analyzed by one-way analysis of variance (ANOVA) and Tukey test or Kruskal-Wallis and Mann-Whitney tests, when appropriate. Correlations between results were evaluated by Spearman's rank (r_s). A difference of $P < 0.05$ was considered to be statistically significant.

RESULTS

The structure of eugenol isolated from hydrolate of *O. gratissimum* was determined by comparison of its mass spectrum, retention index and NMR spectra with data reported in the literature (Adams, 2001; Carrasco et al., 2008; SDBS, 2008). The ethanolic extracts of *O. gratissimum* differed significantly in extractive yield and eugenol content (Table 1). The largest yield was obtained by Soxhlet (19.5 %), which also required a longer extraction time compared with the ultrasound and microwave-assisted methods. The highest eugenol content was obtained by microwave-assisted extraction and maceration, followed by the soxhlet and ultrasonic methods. Although there were no statistical differences in extractive yields between ultrasound methods, UCB extracted more eugenol content than UH.

All extracts tested showed bacteriostatic activity (Table 2), except against *Salmonella* sp. Most of the extracts obtained by the two ultrasound methods had similar MIC values against Gram-positive bacteria to each other and also compared with the classical maceration procedure. The exception was the extract obtained by UCB against *S. aureus* ATCC 25923, which showed the lowest MIC of all extracts. For Gram-negative bacteria, the UH extract was the most effective against extended-spectrum β-lactamase-producing *E. coli* (ESBL) and *S. choleraesuis* ATCC 10708. The extract obtained by microwave extraction was statistically similar to one or both extracts obtained by conventional and ultrasonic processes for *B. cereus* ATCC 14579, *S. aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA), ESBL, *S. flexneri* and *P. aeruginosa* ATCC 27853. Only the conventional techniques generated active extracts against the diarrheagenic pathogens tested, such as *Shigella* sp. and enteropathogenic *Escherichia coli* (EPEC). The extract obtained by maceration gave the best results against MRSA. The extract obtained by maceration showed a lower MIC against Gram-positive bacteria in comparison to the Soxhlet extract. The only exception was vancomycin-resistant *E. faecalis* ATCC 51299 (VRE), which showed more susceptibility to the extracts obtained by Soxhlet and microwave.

The extracts showed bactericidal activity assayed occurred against only some of the Gram-positive bacteria, except for the extract obtained by Soxhlet against *S. flexneri*. The eugenol content in the extracts was positively correlated ($r_S = 0.894$) with the number of bacterial strains with the lowest MBC for each extract (Figure 1A). On the other hand, the eugenol content and the bacteriostatic activity of each extract (through the number of bacterial strains with the lowest MIC) were not significantly correlated by Spearman's test ($P > 0.05$).

The extract obtained by maceration (LC_{50} = 331.3 µg/ml) was the most toxic for brine shrimp, followed by UH, UCB and finally Soxhlet /MAE, in decreasing order of toxicity (Table 1). Negative correlation ($r_S = -0.949$) between brine-shrimp toxicity and the number of bacterial strains with the lowest MIC for extracts obtained by the different processes was observed (Figure 1B).

Table 1. Experimental conditions, extract yield, eugenol composition and toxicity by brine shrimp lethality test (BSL) of ethanoli *gratissimum* L.

Experimental condition	Extraction Methods			
	MAC	SOX	UH	UCB
Time	3×7 days	30 h	1 h	1 h
Temperature (ºC)	Ambient	78	13-44	40
Solvent volume/plant material weight ratio (ml/g)	43.8 (3×14.6)	8.6	10	10
Extract yield (%)	11.8±0.2^{b}	19.5±0.9^{a}	10.2±0.6^{bc}	7.7±0.9^{c}
Eugenol content (%)	11.6±0.09^{a}	10.0±0.05^{b}	4.6±0.04^{d}	7.8±0.04^{c}
BSL				
LC_{50} (µg/mL)	331.3	793.4	456.9	586.5
95% Confidence interval	302.1-363.1	690.9-951.8	413.9-511.4	523.9-655.4

MAC: Maceration; SOX: Soxhlet ; UH: ultrasonic horn; UCB: ultrasonic cleaning bath; MAE: microwave-assisted extraction. Data are pre SEM. Different letters in the rows indicate significant differences between ethanolic extracts of *O. gratissimum* using one-way ANOVA follo 0.05).

Table 2. MIC and MBC (mg/ml) of ethanolic extracts of *Ocimum gratissimum* L. obtained by different extraction methods.

Bacteria	Conc.	Extraction methods			
		MAC	SOX	UH	UCB
Gram-positive					
Bacillus cereus ATCC 14579	MIC	1.32^{b}	2.63^{a}	1.32^{bc}	1.32^{c}
	MBC	2.64^{a}	>2.63	2.64^{a}	1.32^{b}
Bacillus cereus	MIC	0.66^{a}	2.63^{b}	0.66^{ac}	0.66^{c}
	MBC	>2.64	>2.63	>2.64	>2.64
Enterococcus faecalis ATCC 51299	MIC	10.56^{a}	5.28^{b}	10.58^{a}	10.57^{a}
	MBC	>10.56	10.57^{a}	>10.58	10.57^{a}
Staphylococcus aureus ATCC 25923	MIC	1.32^{a}	1.32^{a}	1.32^{a}	0.66^{b}
	MBC	2.64^{a}	2.63^{a}	>2.64	>2.64
MRSA[1]	MIC	0.66^{c}	2.63^{a}	1.32^{abc}	1.32^{b}
	MBC	>2.64	>2.63	>2.64	>2.64

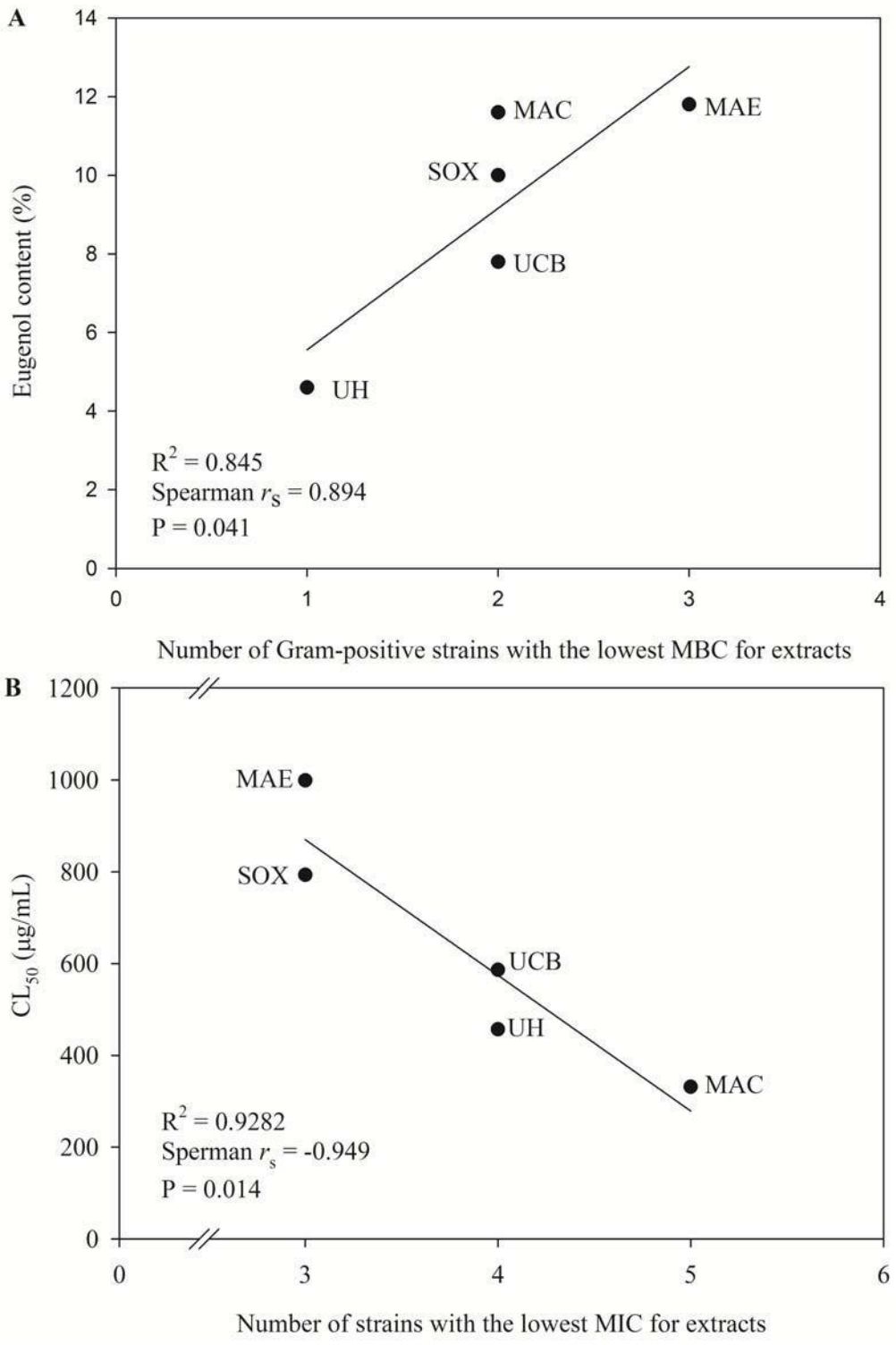

Figure 1. Correlations among results of the ethanolic extracts of *Ocimum gratissimum*. (A) Eugenol content and number of Gram-positive strains with the lowest MBC for extracts; (B) Brine shrimp toxicity and the number of bacterial strains with the lowest MIC for extracts.

(Passos et al., 2009). The differences detected in the bacteriostatic activity of the extracts seem to be a consequence of the synergistic or antagonistic action of eugenol and other constituents, such as oleanolic acid,

flavonoids and caffeic acid esters (Njoku et al., 1997; Grayer et al., 2003). This hypothesis can be partially confirmed by the absence of correlation between the eugenol content and the bacteriostatic activity. However, further studies are required in order to identify the compounds involved in the biological activity of the extracts.

The mechanism that produces the antimicrobial activity of phenolic compounds such as eugenol is not completely understood. In general, these compounds appear to exert their activity at the cytoplasmic membrane through mechanisms such as substrate complexing, membrane disruption, enzyme inactivation and metal chelation (Sikkema et al., 1995; Cowan, 1999; Gill and Holley, 2006; Di Pasqua et al., 2007).

Some bacterial species tested, such as *S. aureus*, *B. cereus*, enterococci, *P. aeruginosa*, *E. coli* and *Salmonella* sp., are common agents of nosocomial infections (Bereket et al., 2012). The development of antibiotic resistance in these pathogens, such as observed in MRSA, VRE and ESBL, and their widespread distribution in community and non-hospital health-care facilities have increased interest in finding new therapeutic alternatives (Lopes, 2005; Gaude and Hattiholli, 2013). Here, the ethanolic extracts of *O. gratissimum* showed different antimicrobial profiles according to the extraction method employed. With respect to VRE, for example, it is possible that the high temperatures used in the microwave and Soxhlet methods favored the extraction of active compounds, or reduced the extraction of constituents with an antagonist effect on antibacterial activity. On the other hand, extracts obtained by ultrasound methods were quite effective against recognized etiologic agents of wound infections, such as *S. aureus*, *B. cereus* and ESBL (Bottone, 2010; Bereket et al., 2012).

All ethanolic extracts of *O. gratissimum* demonstrated bioactivity as assessed by the BSL test (Table 1) according to the classification of Meyer et al. (1982) (LC_{50} < 1000 µg/ml). In view of the relatively low LC_{50} value detected for eugenol (LC_{50} = 186.1 µg/ml) (Silva et al., 2010), the BSL results suggest that this compound in the mixture showed lower toxicity due to a reduction of its concentration. It is also possible that the effect of other constituents could have increased the toxicity of the extracts obtained by maceration and ultrasound processes. A similar correlation between the BSL test and antibacterial results was previously suggested for species of Euphorbiaceae with antimicrobial activity (MacRae et al., 1988), but this correlation was confirmed statistically for the first time in the present study.

Conclusion

The methodology used to produce ethanolic extracts of *O. gratissimum* could be chosen according to their intended use. Ultrasound methods yield extracts that are efficient topical antiseptics. To treat nosocomial infections caused by VRE, the most active extracts could be obtained by microwave-assisted extraction.

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Conflict of interest

The authors report no declarations of interest.

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