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EFFECTS OF *MYRCIA OVATA* CAMBESS. ESSENTIAL OIL ON PLANKTONIC GROWTH OF GASTROINTESTINAL MICROORGANISMS AND BIOFILM FORMATION OF *ENTEROCOCCUS FAECALIS*

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ABSTRACT

The essential oil from the leaves of *Myrcia ovata* Cambess., commonly used in Brazil for the treatment of gastric illnesses, was screened for antimicrobial activity and action in the formation of microbial biofilms by *Enterococcus faecalis*. The oil was obtained by hydrodistillation using a clevenger-type system. Its chemical composition was analyzed using GC and GC-MS. Both MIC and MBC of the essential oil were determined by broth microdilution techniques and agar dilution method. The essential oil showed antimicrobial activity against *E. faecalis, Escherichia coli, Pseudomonas aeruginosa, Salmonella choleraesuis, Staphylococcus aureus, Streptococcus pneumoniae* and *Candida parapsilosis.* The results showed that the essential oil of *M. ovata* Cambess. was effective against the formation of biofilm by *E. faecalis* when compared with the control. Four volatile compounds, representing 92.1 % of the oil, were identified and geranial was the major component (50.4 %). At the best of our knowledge, this is the first report of the chemical composition and antimicrobial activity of the essential oil from leaves of *M. ovata*.

Key words: Essential oil, *Myrcia ovata* Cambess., antimicrobial activity, biofilms.

INTRODUCTION

The gastrointestinal tract features several diverse "macro" environments, including the oral cavity, the stomach, the small intestine, and the large intestine (colon). The density of bacteria along the GI tract can vary greatly and this wide diversity of “macro” and “micro” environments harbors an even wider diversity of bacterial species, and an estimated 500 to 1000 different species are present in the GI tract. Recently there is an understanding about immune-mediated biofilm formation by commensal bacteria in the mammalian gut, and on biofilm distribution in the large bowel (7).

Plants have been used as medicines over hundreds of years and constitute an obvious choice for study (15). It is interesting to determine whether their traditional uses are supported by actual pharmacological effects or merely based on folklore.

Essential oils, which are extracted from plants (e.g. leaves, peels) have also been used for many years to treat a variety of medical ailments, although the mechanism of their action is

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generally not clarified (15).

Myrcia ovata Cambess., popularly known as laranjinha-do-mato, is a shrub about 8 m tall that belongs to the Myrtaceae family that presents about 140 gender, with approximately 3000 species. Its leaves are commonly used as tea, in folk medicine, for the treatment of gastric illnesses, gastritis and diarrhea. Some species of *Myrcia* are used in the popular medicine, standing out *Myrcia fallax* and *Myrcia multiflora* (19).

Despite the popular use of *M. ovata* as medicinal plant, there are no data about the antimicrobial effect and phytochemical profile from the leaves of this vegetal specimen. Thus, the interest of this plant is justifiable because of its potential medicinal value.

The aim of this study was to examine the antimicrobial and antifungical activities of the essential oil of *M. ovata*, on some GI representative strains, and its chemical composition. No reference to any previous analysis about the essential oil of *M. ovata* could be found. In addition, it was studied *M. ovata* action in microbial biofilms of *Enterococcus faecalis* one of the major antimicrobial resistant component of the GI tract that is known to participate as biofilm in numerous chronic man infection.

MATERIALS AND METHODS

Plant Material

The fresh leaves of *M. ovata* were collected in October 2004, at the flowering stage, in Guaramiranga - Ceará State (Northeast of Brazil), after its identification by botanists of the Department of Biology, Federal University of Ceará. A voucher specimen was deposited (#039558) at Prisco Bezerra Herbarium of the same university.

Essential oil extraction and analysis

The fresh leaves, 1.0 Kg of *M. ovata*, were cut in small pieces and hydrodistilled for 2h, in a clevenger-type glass hydrodistillation apparatus, to produce oil in 0.9 % yields on fresh weight basis. The sample oil, which had a pale yellow color, was dried over sodium sulfate and stored in sealed glass vials at low temperature before analysis (4).

The essential oil from aerial parts of *M. ovata* was analyzed using GC and GC-MS. GC analysis was performed on a Shimadzu GC-17A gas chromatograph equipped with flame ionization detector using a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). Hydrogen was used as carrier gas at a flow rate of 1 ml/min and 30 psi inlet pressure; split ratio 1:30. The column temperature was programmed from 35 ºC to 115 ºC at a rate of 4 ºC/min, then heated at a rate of 17 ºC/min to 280 ºC and held isothermal for 10 min; both injector temperature and detector temperature were 250 ºC. The GC-MS analysis was carried out on a Hewlett-Packard Model 5971 GC/MS using a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness); carrier gas helium, flow rate 1 ml/min and with split mode. The injector temperature and detector temperature were 250 ºC and 200 ºC, respectively. The column temperature was programmed from 35 °C to 180 ºC at 4 ºC/min and then 180 °C to 250 ºC at 10 ºC/min. Mass spectra were recorded from 30 – 450 m/z. Individual components were identified by matching their 70 and V mass spectra with those of the spectrometer data base using the Wiley L-built library and other two computer libraries MS searches using retention indices as a preselection routine as well as by visual comparison of the fragmentation pattern with those reported in the literature (3, 4, 10, 25).

Microorganisms used and growth conditions

Representative microorganisms for gastric and GI disorders were included in this study. The test organisms included the bacteria *E. faecalis* ATCC 29212, *Escherichia coli* ATCC 25992, *Helicobacter pylori* TX30A, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella choleraesuis* ATCC 10708, *Staphylococcus aureus* ATCC 25923, *Streptococcus pneumoniae* ATCC 49619 and the yeast *Candida parapsilosis* ATCC 22019. Microorganisms were obtained from the Brazilian Culture Collections from the Oswaldo Cruz Foundation (FIOCRUZ). The *H. pylori* reference strain

(TX30A) was obtained from the collection of the Dr JC Atherton, Vanderbilt University, Nashville, Tennessee, USA. Bacterial strains were cultured overnight at 37º in Brain Heart infusion (BHI) agar (Oxoid) supplemented with 5 % sheep blood and maintained frozen at 4 ºC in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with 20 % glycerol. The *S. pneumoniae* strain was cultured on Columbia agar (DIFCO) supplemented with 5% defibrinated sheep blood and 5µg/ml of gentamycin (Sigma Chemical Co., St. Louis) and incubated in 5 % CO_2 at 36 ºC by 24 h. The maintenance of the bacterial strain was accomplished in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with 20 % glycerol at 4 ºC (18). The *Helicobacter pylori* strain was cultured on Belo Horizonte medium plates (BHM) at 37 ºC under microaerophilic conditions and maintained frozen at -80 ºC in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) with 20 % glycerol. The Belo Horizonte medium was prepared with Brain Heart infusion (BHI) agar (Oxoid) supplemented with 10 % sheep blood and containing a final concentration of 6 mg of vancomycin (Sigma Chemical Co., St. Louis, Mo) per liter, 20 mg of nalidixic acid (Sigma Chemical Co., St. Louis, Mo) per liter, 500 mg de cycloeximide (Sigma Chemical Co., St. Louis, Mo) per liter and 40 mg of 2,3,5-triphenyltetrazolium chloride (TTC) (Riedel, De Haën, AG Seelze, Hannover, Federal Republic of Germany) per liter (22). Yeast was cultured overnight at 30 ºC and maintained in Potato agar (Acumedia Manufactures).

Inhibitory-zone testing

Suspensions of fresh cultures of each tested microorganisms were made in saline and turbidity adjusted to 3x108 CFU/ml (*Helicobacter pylori*) or 1.5x108 CFU/ml (others bacteria and yeast) corresponding to McFarland standards 1 and 0.5, respectively. A volume of 0.1 ml of each tested bacterial suspension was spread onto a brain heart infusion agar (BHI agar) containing 5 % (v/v) defibrinated sheep blood using a sterile cotton swab in order to get a uniform microbial growth on both control and tests plates. Wells of 7 mm in diameter were punched on the plates and 30 Tl of the essential oil of *M. ovata* was incorporated into the wells. DMSO was used as control. The plates were incubated at 37 ºC under microaerophilic conditions for 48 h of *H.pylori* and at 37 ºC for 24 h for the other bacteria (6). Yeast was incubated at 30 ºC for 24 h. The clear zone around each well was observed and its diameter was examined (8).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) of essential oil were determined by broth microdilution techniques for *E. faecalis, E. coli, P. aeruginosa, S. choleraesuis, S. aureus, S. pneumoniae and C. parapsilosis* using twofold increments (0.0078-1 %) (8, 9). MIC of essential oil for *Helicobacter pylori* was determined by the agar dilution method, using twofold increments (2.4–12.5 mg/ml), on BHI agar plus 10 % sheep blood and 0.004% 2,3,5-triphenyltetrazolium chloride (9). Tetracycline (1 mg/ml) and anfotericin B (16 µg/ml) were the positive control for bacteria and yeast, respectively.

Determination of minimum bactericidal concentration (MBC)

Referring to results of the MIC assay, the wells showing complete absence of growth were identified and 5 µl of each well were transferred to agar plates (BHI and SDA) and incubated at previously-mentioned times and temperatures. The complete absence of growth was considered as the minimum bactericidal concentration (15).

Biofilm susceptibility assay

Single species biofilms of *E. faecalis* were generated on sterile cellulose nitrate membrane filters (CNM) (0.2 µm pore size, 13 mm diameter; Millipore Industrial e Commercial LTDA). The inoculum was prepared in a liquid medium following conventional methodology (9). A volume of 50 µl of fresh bacterial growth in BHI broth and adjusted to the turbidity of 6 McFarland (optical density at 600 -2.27 nm) was inoculated onto the membranes placed on BHI agar blood plates (5 % defibrinated sheep blood) and incubated for 24 hours aerobically. The bacterial suspension (50 µl) was

inoculated onto the membranes placed on BHI agar blood plates and incubated aerobically for 8 days. Each three days the BHI agar blood plates were changed. After the incubation time of 8 days membrane filters were removed aseptically from the agar plate and transferred carefully, so as to avoid the disruption of the biofilm, into 5 ml of the essential oil (0.031, 0.125, e 0.5 %) and incubated for 5, 10 and 30 min. The three times were used for the three concentrations of the tested essential oil. Sterile saline was used as a control group. After the designated contact time, the membrane filters were then carefully transferred to 5 ml of neutralizing broth (D/E Neutralizing Broth, Acumedia Manufactures, Michigan 48912) to stop the antimicrobial action of the test agent and vortexed for 30 s to re-suspend the organisms. Ten-fold serial dilutions were generated in D/E Broth. Each dilution was plated in triplicate onto D/E agar plates. The plates were then incubated for 24/48 h in aerobiosis at 35 °C and colony forming units (CFU) was calculated (2).

Statistical Analysis

Statistical analysis was performed SPSS for Windows versão 15.0. Differences among means groups were analyzed by the Student's t-tests. A P-value < 0.05 was considered significant. The means and standard deviation were calculed.

RESULTS

Plant essential oils have been used for many thousands years, in food preservation, pharmaceuticals, alternative medicine and natural therapies. It is necessary to investigate those plants scientifically which have been used in traditional medicine to improve the quality of healthcare. Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens (23). In vitro studies in this work showed that the essential of *M. ovata* inhibited bacterial and fungal growth but their effectiveness varied.

In our study, a total of 7 microorganisms were tested and the results are summarized in Table 1, following by the Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in Table 2. As showed in Table 1, the essential oil showed antimicrobial activity against some microorganisms tested, however, it did not show antimicrobial activity against *H. pylori*. In the assays against microorganisms by inhibitory zone testing, the zones obtained were between 16 to 36 mm. *E. faecalis* and *C. parapsilosis* were the most sensitive microorganisms. The MIC values obtained ranged between 0.0625–1 % (Table 2). The best results were observed against *E. faecalis* and *S. pneumoniae*. The MBC values obtained were similar to the observed in MIC for *E. faecalis, E. coli, P. aerugionosa* and *S. choleraesuis* and superior for *S. aureus* and *S. pneumoniae.*

Table III shows the identified constituents and their percentage composition, as well as their retention indices (RI) values listed in order of elution from a DB-5 capillary column. GC/MS and GC/FID analysis of the essential oil from leaves of *M. ovata* identified four phytochemicals as constituents, representing 92.1% of the oil, of these geranial was the major compound (50.4%) followed neral (35.8%), 1,8-cineole (4.8%) and α-terpineol (1.1%). The chemical composition of the essential oil was essentially constituted by monoterpenes.

Table 1. Antimicrobial activity of the essential oil of *Myrcia ovata* Cambess.

Microorganisms	Diameter of inhibition zone (mm)
Enterococcus faecalis (ATCC 29212)	36
Escherichia coli (ATCC 25992)	21
Helicobacter pylori (TX30A)	0
Pseudomonas aeruginosa (ATCC 27853)	16
Salmonella choleraesuis (ATCC 10708)	26
Staphylococcus aureus (ATCC 25923)	23
Streptococcus pneumoniae (ATCC 49619)	25
Candida parapsilosis (ATCC 22019)	30

ATCC (American Type Culture Collection)

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) exhibited by the essential oil of *Myrcia ovata* Cambess.

Microorganisms	MIC (%)	MBC (%)
Enterococcus faecalis (ATCC 29212)	0.031	0.031
Escherichia coli (ATCC 25992)	1	1
Helicobacter pylori (TX30A)	0	0
Pseudomonas aeruginosa (ATCC 27853)	> 1	> 1
Salmonella choleraesuis (ATCC 10708)	0.5	0.5
Staphylococcus aureus (ATCC 25923)	0.25	0.5
Streptococcus pneumoniae (ATCC 49619)	0.0625	0.25
Candida parapsilosis (ATCC 22019)	0.031	-

ATCC (American Type Culture Collection)

Table 3. Chemical composition (%) of the essential oil from leaves of *Myrcia ovata* Cambess. (Myrtaceae).

Constituent	RI	Leaf Oil (%)
1,8-Cineole	1031	4.8
α-Terpineol	1189	1.1
Neral	1238	35.8
Geranial	1267	50.4
TOTAL		92.1

RI = Retention index

DISCUSSION

E. faecalis is an important opportunistic pathogen having the ability to adhere to biotic and abiotic surfaces allowing the formation of a biofilm that permits to grow and survive in harsh environments prevailing in the root canal (12, 14, 17). A number of infections caused by *E.faecalis* strains have proven to be particularly difficult to treat with current antibiotic therapies, partly owing to their intrinsic antibiotic resistance elements, capacity of growing in a biofilm (24). It has gained notoriety as a persistent organism that can survive as a monoculture in root canals (20). It has been reported that persistent endodontic infections are often caused by *E. faecalis* biofilms (11).

The results showed that essential oil of *M. ovata* was the effective agent against *E. faecalis* biofilm with bacterial reduction after 5, 10 and 30 min exposure to essential oil in the three concentrations analyzed being compared with the control. The tested concentrations were chosen in agreement with the result obtained in MIC accomplished for *E. faecalis* strain. The numbers of CFU after exposure of *E. faecalis* biofilm were counts using the natural logarithm to provide a normally distributed outcome. The Student´s t-test showed difference among means groups with P-value <0.01. 10 min incubation of biofilm with the essential oil 0.5 % it was the time of larger reduction of strain, compared with control.

Because the antibiotic-resistant *E. faecalis* biofilm, the search for safe and effective new agents is important. The increased resistance conferred by the biofilm is thought to result from a number of factors including phenotypic

differences in sessile bacteria compared to planktonic bacteria, differential rates of bacterial metabolism at various sites within the biofilm, and matrix inhibition of diffusion of charged antimicrobial agents (13). The tradicional use and anecdotal evidence of plants as medicine provide the basis for suggesting the essential oils and plant extracts may be useful for medical conditions. In this study, we showed that essential oil of *M. ovata* had bactericidal effect against *E. faecalis* biofilm and several bacteria beyond the antifungal effect.

Several papers published have dealed with antimicrobial activity (1, 5, 16, 21, 26) of geranial and neral. Therefore, these major components of the essential oil from leaves of *M. ovata* can be the responsible for the activity. It will be necessary to examine each component of essential oil separately, and in combination to ascertain whether they act alone or synergistically.

We believe that the present investigation provide support to the antibacterial and antifungal properties of essential oil of *M. ovata*. In can be used as supplement in the developing countries towards the development of new therapeutic agents. Additional in vivo studies would be needed to justify and further evaluate the potential of oil as an antibacterial agent in topical or oral applications.

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