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PROGRAMA DE PÓS-GRADUAÇÃO STRICTO SENSU EM CONSERVAÇÃO E MANEJO DE RECURSOS NATURAIS – NÍVEL MESTRADO

LAÍS DAYANE WEBER

COMPOSIÇÃO QUÍMICA, ATIVIDADE BACTERIANA E ANTIOXIDANTE DE ÓLEO ESSENCIAL E DIFERENTES EXTRATOS VEGETAIS DE *Prunus myrtifolia* (L.) Urb.

CASCAVEL-PR

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Dissertação apresentada ao Programa de Pós-graduação *Stricto Sensu* em Conservação e Manejo de Recursos Naturais – Nível Mestrado, do Centro de Ciências Biológicas e da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para a obtenção do título de Mestre em Conservação e Manejo de Recursos Naturais.

Área de Concentração: Conservação e Manejo de Recursos Naturais

Orientadora: Fabiana Gisele da Silva Pinto

CASCAVEL-PR

Novembro/2013

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por todo amor e constante incentivo.

‘"Apesar dos nossos defeitos, precisamos enxergar que somos pérolas únicas no teatro da vida e entender que não existem pessoas de sucesso e pessoas fracassadas. O que existe são pessoas que lutam pelos seus sonhos ou desistem deles." (Augusto Cury)

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SUMÁRIO

RESUMO

A propriedade antimicrobiana das plantas pode ser explicada pela produção de compostos ativos gerados durante o metabolismo secundário como também por compostos voláteis. Atualmente, os conhecimentos desta propriedade têm sido confirmados cientificamente, revelando assim o enorme potencial das plantas no controle de doenças infecciosas, enquanto verifica-se um aumento nos casos de micro-organismos patogênicos resistentes aos antimicrobianos conhecidos. Extratos e óleos essenciais de plantas têm mostrado efeitos sobre desenvolvimento de micro-organismos em inúmeras situações, o que sugere uso prático destes produtos. No presente estudo voltado à pesquisa de plantas como fonte natural e alternativa de substâncias antimicrobianas, determinou-se a composição química do óleo essencial e de diferentes extratos vegetais (aquoso, etanolico, acetato de etila e hexânico) de *Prunus myrtifolia* (L.) Urb. (pessegueiro-bravo), através da CG/MS e triagem fitoquímica respectivamente, bem como seu efeito antimicrobiano contra micro-organismos Gram negativos *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* Typhimurium (ATCC 14028), *Proteus mirabilis* (ATCC 25933), *Klebsiella pneumoniae* (ATCC 13883) e *Escherichia coli* (ATCC 25922), Gram positivos como, *Enterococcus faecalis* (ATCC 19433), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 25923) e *Bacillus subtillis* (CCCD - B005) e como levedura a *Candida albicans* (ATCC 10231) através da determinação dos valores de Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM) utilizando a técnica de microdiluição em caldo; e por fim buscou-se avaliar a atividade antioxidante do óleo essencial e dos extratos vegetais pelo método de captura de radicais livres DPPH (2.2difenil-1-picril-hidrazil). A maior classe de compostos voláteis identificados no óleo de *Prunus myrtifolia* foi benzaldeido (97%) seguido de 3-hexen-1-ol (0.07%) e benzoato de benzila (0.09%). De maneira geral através da triagem fitoquimica dos extratos verificou-se a presença de metabolitos secundários como, flavonoides, taninos (etanolico e aquoso), triterpenoides e saponinas (etanolico), que já se mostraram ativas em diferentes estudos descritos na literatura. Em relação ao extrato hexânico apresentou ausência de metabólitos secundários com atividade antimicrobiana. Os resultados apontam o extrato aquoso e etanolicos como os mais efetivos os patógenos testados. Em relação ao óleo, apresentou atividade antimicrobiana frente a todos patógenos avaliados. Em uma terceira etapa do estudo verificou-se atividade antioxidante entre o extrato aquoso, etanolico e acetato de etila; em relação ao óleo essencial e o extrato hexânico não foi detectada atividade antioxidante. Pelos resultados obtidos ficou estabelecida a capacidade antimicrobiana dos produtos vegetais testados, bem como determinou-se a atividade antioxidante dos mesmos. Em segunda etapa da pesquisa realizou-se Avaliou-se o perfil fitoquímico, ação antioxidante e antimicrobiana dos extratos vegetais etanólico e aquoso de seis plantas brasileiras obtidos das folhas secas de *Maytenus aquifolia* Mart. (espinheira-santa), *Plinia cauliflora* (Mart.) O. Berg (jabuticabeira), *Ocotea spixiana* (Nees) Mez. (canela-branca), *Psidium guajava* L. (goiabeira), e *Ricinus communis* L. (mamona) e *Schinus molle* L. (aroeira). A atividade antimicrobiana in vitro dos extratos vegetais foi testada frente a trinta e seis sorotipos de *Salmonella* de origem avícola pelo método de microdiluição em caldo com a determinação da Concentração Inibitória Mínima (CIM) e a Concentração Bactericida Mínima (CBM). A ação antioxidante dos mesmos foi avaliada pelo método de DPPH (2,2-difenil-1-picril-hidrazila). O perfil fitoquímico detectou componentes com potencial

antimicrobiano e antioxidante em todos os extratos, assim como um percentual de captura do DPPH superior a 65%, demonstrando o elevado potencial antioxidante dos extratos testados. Nos testes de microdiluição em caldo, observou-se a atividade antimicrobiana de todos os extratos testados, sendo que em geral os extratos etanólicos foram mais eficazes quando comparados aos aquosos, sendo o extrato etanólico de *P. cauliflora* seguido por *P. guajava* de maior efeito bacteriostático. As CIMs variaram entre 1,56-100 $mg.mL^{-1}$ e a CBM entre 3,13-100 $mg.mL^{-1}$. Esses resultados confirmaram o potencial antimicrobiano e antioxidante desses extratos vegetais.

Palavras-chave: Ação antimicrobiana, Plantas nativas, Patógenos, Extrato Vegetal, Óleo Essencial.

ABSTRAT

The antimicrobial property of the plants can be explained by the production of active compounds generated during secondary metabolism as well as volatile compounds. Currently, the knowledge of this property have been confirmed scientifically, thus revealing the enormous potential of the plants in the control of infectious diseases, while there is an increase in cases of pathogenic microrganisms resistant to known antibiotics. Essential oils and extracts of plants have shown effects on growth of micro -organisms in many situations, suggesting practical use thereof. In the present study focused on the research of plants as alternative and natural source of antimicrobial substances, determined the chemical composition of the essential oil and various plant extracts (aqueous, ethanolic, ethyl acetate and hexane) of *Prunus myrtifolia* (L.) Urb. by GC/MS and phytochemical screening respectively, and its antimicrobial effect against microorganisms Gram negative *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Proteus mirabilis* (ATCC 25933), *Klebsiella pneumoniae* (ATCC 13883) and *Escherichia coli* (ATCC 25922) as Gram positive *Enterococcus faecalis* (ATCC 19433), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (CCCD - B005) and yeast such as *Candida albicans* (ATCC 10231) by determining the values of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using the microdilution broth; and finally we sought to evaluate the antioxidant activity of essential oil and plant extracts by the capture of free radicals DPPH (2.2difenil-1-picryl-hydrazyl). The largest class of volatile compounds identified in the oil was *Prunus myrtifolia* benzaldehyde (97%) followed by 3-hexen-1-ol (0.07 %) and benzyl benzoate (0.09 %). Generally through the phytochemical screening of the extracts was found the presence of secondary metabolites such as, flavonoids, tannins (ethanolic and aqueous), and triterpenoid saponins (ethanolic), which have proven active in different studies in the literature. Compared to hexane extract showed absence of secondary metabolites with antimicrobial activity. The results indicate the aqueous and ethanolic extract as the most effective of the tested pathogens. Regarding oil, showed antimicrobial activity against all pathogens evaluated. In a third stage of the study it was found antioxidant activity of the aqueous extract, ethanolic and ethyl acetate; in relation to essential oil and hexane extract antioxidant activity was not detected. From the results obtained it was established antimicrobial capacity of plant products tested and determined the antioxidant activity of the same . In the second stage of the research took place evaluated the phytochemical profile , antioxidant and antimicrobial activity of ethanolic and aqueous plant extracts from six Brazilian plants obtained from the dried leaves of *Maytenus aquifolia* Mart., *Plinia cauliflora* (Mart.) O. Berg, *Ocotea spixiana* (Nees) Mez., *Psidium guajava* L., *Ricinus communis* L. and *Schinus molle* L. The in vitro antimicrobial activity of plant extracts was

tested against 36 serotypes of *Salmonella* from poultry products by the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The antioxidant properties of these was evaluated by DPPH (2.2-diphenyl-1-picryl-hidrazila) method. The phytochemical profile detected components with antimicrobial and antioxidant potential in all extracts , as a percentage capture of DPPH than 65 % , demonstrating the high antioxidant activity of the tested extracts. In microdilution tests, we observed the antimicrobial activity of all tested extracts , and in general the ethanol extracts were more effective when compared to aqueous and ethanol extract of *P. cauliflora* followed by *P. guajava* higher end bacteriostatic . The MIC ranged from 1.56 to 100 $mg.mL^{-1}$ and MBC of 3.13 to 100 $mg.mL^{-1}$. These results confirmed the antioxidant and antimicrobial potential of these plant extracts.

Keywords: Antimicrobial Action, Native Plants, Pathogens, Plant Extract, Essential Oil.

CAPÍTULO 1:

Chemical Composition and Antimicrobial and Antioxidant Activity of Essential Oil and Various Plant Extracts from *Prunus myrtifolia* (L.) Urb

O artigo segue as normas sugeridas pela revista “African Journal of Agricultural Research” citada em Anexos Capítulo 1

Cascavel, 2013

Chemical Composition and Antimicrobial and Antioxidant Activity of Essential Oil and Various Plant Extracts from *Prunus myrtifolia* (L.) Urb

Laís Dayane Weber[1]*; Fabiana Gisele da Silva Pinto[1]; Mayara Camila Scur[1]; Juliete Gomes de Lara de Souza[1]; Willian Ferreira da Costa[2]; Camila Wihby Leite[2].

[1] Biotechnology Laboratory, West of Paraná State University, Cascavel, PR, Brazil.

[2] Department of Chemistry, State University of Maringá, Maringá, PR, Brazil.

ABSTRACT

In this study focused on research on plants as a source of alternative and natural antimicrobial substances, the chemical composition of the essential oil from *Prunus myrtifolia* (L.) Urb. was assessed through gas chromatography coupled to mass spectrometry (GC/MS) and phytochemical screening of different extracts (aqueous, ethanolic, ethyl acetate, and hexanic) from the same plant, as well as the antimicrobial effect against the following microorganisms: *Pseudomonas aeruginosa; Salmonella* Typhimurium; *Proteus mirabilis; Klebsiella pneumoni; Escherichia coli; Enterococcus faecalis*; *Staphylococcus epidermidis; Staphylococcus aureus; Bacillus subtilis* and *Candida albicans*, through determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, using the micro-dilution broth method. Finally, the goal was to assess the antioxidant activity of essential oil and plant extracts using the DPPH free radical method (2.2-diphenyl-1-picrylhydrazyl). The largest class of volatile compounds identified in *P. myrtifolia* oil belongs to aldehydes represented by benzaldehyde compounds. With respect to antimicrobial activity, all extracts and essential oil showed activity against the microorganisms assessed, with exception of hexanic extract. Among the extracts assessed, aqueous and ethanolic extracts were the most effective. Antioxidant activity of aqueous, ethanolic and ethyl acetate

extracts was confirmed; however, antioxidant activity of essential oil and hexanic extract was not observed.

Keywords: antimicrobial activity, GC/MS, native plants, chemical composition, antioxidant activity, essential oil, plant extracts

INTRODUCTION

Brazil has the largest equatorial and humid tropical forest on the planet and, consequently, little explored extensive plant genetic diversity. With respect to the medicinal potential, only approximately 17% of plants have been studied (Pinto et al., 2002). Exploration of these plants is required, because potentially useful compounds can be lost due to the extinction of some species (Patinõ and Cuca, 2011). Due to this diversity, Brazil came to prominence in the search for potential bioactive compounds that can be used for various purposes, such as alternative antimicrobial products for controlling pathogens (Pupo et al., 2007) used in the pharmaceutical and food industries (Cehyan et al., 2012).

The family Rosaceae comprises around 100 genera and 3000 species. Concentrated in the northern hemisphere, it is one of the leading families from an economic point of view, showing a few native species in Brazil (Souza and Lorenzi, 2005). Some species have great pharmacological and nutrition potential and are used in popular medicine for the treatment of various diseases and for the maintenance of good health. The genus *Prunus* is composed of approximately 130 species that occur in the northern, southern and southeastern regions of Brazil. Various fruits introduced and consumed in Brazil belong to this genus, such as peaches (*P. persica*), nectarines (*P. persica* var. nucipersica), plums (*P. domestica*), almonds (*P. dulcis*), and cherries (*P. avium*, *P. cerasus*) (Souza and Lorenzi, 2005).

Regarding Brazilian native species of the genus *Prunus*, the species *Prunus myrtifolia* (L.) Urban deserves attention. For being a species of wide geographic distribution, it is synonymous with *P. sphaerocarpa* Hook and *P. sellowii* Koehne (Souza and Lorenzi, 2005).

In recent years, the chemical compositions as well as the antioxidant and antimicrobial properties of plants have gained interest in the search for alternative products. Essential oils can contain from 20 to 60 (or more) diverse compounds and in the most varied concentrations (Bakkali et al., 2008). The analysis requires the application of current analytical methods and adapted instrumentation, which allows assessing the quality of essential oils and ensure the identification of their constituents. Plant extracts are targets of great interest due to the presence of secondary metabolites in their composition, which are substances used against pathogenic microorganisms, insects and herbivorous animals. In addition, they have a varied chemical composition with the presence of terpenoids, alkaloids and coumarins, which often feature antimicrobial activity (Reschke et al., 2007).

With the progressive development of synthetic antimicrobial resistance, the biological properties of plant products have been studied in search of alternative products with antimicrobial action (Arya et al., 2010). In this context, essential oils and plant extracts stand out as efficient antimicrobials (Bona et al., 2010).

The search for new natural antioxidants has increased and led food, cosmetics and pharmaceutical industries to focus their searches on materials of plant origin. Plant antioxidants are very varied, but the phenolic compounds have been considered responsible for greater antioxidant capacity, being represented by flavonoids and isoflavones, tannins, lignans, and xanthones, among others (Razavi et al., 2008).

The goal of this study was to determine the chemical composition of the essential oil and various plant extracts from *P. myrtifolia*, as well as their antimicrobial effect against different microorganisms, such as: *Pseudomonas aeruginosa* (ATCC 27853); *Salmonella* Typhimurium (ATCC 14028); *Proteus mirabilis* (ATCC 25933); *Klebsiella pneumoniae*

(ATCC 13883); *Escherichia coli* (ATCC 25922); *Enterococcus faecalis* (ATCC 19433); *Staphylococcus epidermidis* (ATCC 12228); *Staphylococcus aureus* (ATCC 25923); *Bacillus subtilis* (CCD-04) and *Candida albicans* (ATCC 10231). Finally, we aimed to assess the antioxidant activity of the essential oil and plant extracts.

MATERIAL AND METHODS

Plant material

The leaves of *P. myrtifolia* were collected in the western region of the State of Parana, Brazil (24°57' S - 53°28' W), in January and February 2013. The material was identified and incorporated into the Herbarium of the West of Parana State University (UNOP) under number 25 J. Silva, J. P. B.

The leaves collected were dried in an oven with air circulation at 40°C for 48 hours and subsequently ground using a cutting mill with less than 0.42 mm granulometry. The plant material ground was stored protected from the light until its use for the production of extracts.

Obtaining aqueous extract (W)

We added 20 g of the ground plant material to a container with distilled water that was kept in a rotary shaker at 220 x g for 24 hours. Subsequently, the material was filtered in filter paper (Whatman Nº 1) and centrifuged at 5000 x g for 15 minutes. The supernatant material was collected and the final concentration was 200 mg/mL. The extract was stored at 4°C until use.

Obtaining of organic extracts

The organic extracts were obtained according to the methodology described by Ceyhan et al. (2012) with modifications. Ethanol (95%), ethyl acetate and hexane were used as organic solvents. Starting with 10 g, the ground plant material was added to 100 mL organic solvent and placed in a rotary shaker at 220 x g for 24 hours. Subsequently, it was filtered in filter paper (Whatman Nº 1) and centrifuged at 5000 x g for 15 min. The supernatant material was

collected and submitted to roto-evaporation in order to remove the solvent. The extract obtained was diluted at a concentration of 150 mg/mL for ethanolic extracts (ET) and ethyl acetate (EA) and at a concentration of 6 mg/mL for hexanic extract (H) with 10% dimethyl sulfoxide (DMSO), following the proportion of its weight and volume. The extracts obtained were stored at 4°C until use.

Phytochemical screening

The main secondary metabolites were detected in accordance with the methodology developed by Matos, 1997.

Essential oil extraction (EO)

Nearly 70 g of fresh leaves of *P. myrtifolia* in 600 mL distilled water were submitted to standard water steam dragging methodology for three hours using Clevenger-type equipment. The oil was collected directly with no addition of solvent and stored at 4°C.

Chemical composition analysis

The constituents of the essential oil were identified through gas chromatography coupled to mass spectrometry (GC-MS) and the determination of their Kovats retention index (KI).

GC-MS

Analysis of oil from *P. myrtifolia* was carried out using a Thermo-Finnigan GC-MS system, composed of a FOCUS GC gas chromatograph (Thermo Electron), coupled to a DSQ II mass spectrometer (Thermo Electron) and a TriPlus AS automatic injector (Thermo Electron). Chromatographic separation was performed with an HP-5ms fused silica capillary column (30 m long, 0.25 ID and 0.25 µm film; composition of 5% phenyl-95% dimethylpolysiloxane).

The temperature of the injector was 250°C. Samples and patterns of alkanes were injected using the split mode with a split ratio of 1:25. The programming of the temperature used was: 50°C maintained for 2 min; temperature rise to 180°C at a ratio of 2°C min-1; followed by an increase to 290°C at a ratio of 5°C min-1. The interface between the GC and MS was maintained at 270°C and the temperature of the ionization source of the mass spectrometer

was 250°C. The identification of the components was performed by comparing their retention times with those obtained in the literature (Adams, 2007) for the same compounds analyzed by means of Kovats retention index.

Microorganisms used

To perform the antimicrobial activity test of the essential oil and plant extracts from *P. myrtifolia*, we used 5 gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853; *Salmonella* Typhimurium ATCC 14028; *Proteus mirabilis* ATCC 25933; *Klebsiella pneumoniae* ATCC 13883; and *Escherichia coli* ATCC 25922), 4 gram-positive bacteria (*Enterococcus faecalis* ATCC 19433; *Staphylococcus epidermidis* ATCC 12228; *Staphylococcus aureus* ATCC 25923; and *Bacillus subtilis* CCCD-B005) and *Candida albicans* ATCC 1023 as yeast.

Microorganisms previously kept at -20°C were recovered in enriched medium (Brain Heart Infusion) and incubated at 36°C for 24 hours. After this period, they were ressuspended in 0.9% sterile saline solution to obtain the standard inoculum at a concentration of 1×10^8 UFC/mL on the MacFarland scale. Subsequently, dilutions were performed in 0.9% sterile saline solution in order to obtain a final inoculum at a concentration of 1×10^5 UFC/mL, with the exception of *C. albicans* that was used at the final concentration of 1×10^6 UFC/mL.

Determination of minimum inhibitory concentration (MIC)

Essential oil

The MIC of the essential oil was determined using the broth microdilution method. We used 96-well plates, according to the CLSI document M31-A317 with modifications. We added 200 µl of EO from *P. myrtifolia*, at a concentration of 7000 µg/mL with Mueller-Hinton broth (MH) for bacteria and RPMI for yeast in the first well and, after homogenization, successive dilutions were held, obtaining final concentrations from 7000 to 13.67 µg/mL. Aliquots (10 µl) of microorganisms' dilution were distributed in each well containing the EO in its final dilutions. The plates were incubated at 36°C for 24 hours. After turbidity was observed, each

well received an aliquot of 10 µl of 0.5% triphenyl tetrazolium chloride (TTC). After three more hours of incubation at 36°C, the MIC was defined as the lowest concentration of oil in µg/mL able to prevent microbial growth (Sartoratto et al., 2004).

Plant extracts

The MIC of extracts was determined using the broth microdilution method proposed by Ayres et al. (2008) with modifications. Aliquots (10 µl) of dilution were distributed in 96-well microtitre plates, containing 150 µl of MH broth (double concentration) for bacteria and RPMI for yeast, with the previous addition of extracts. The extracts were diluted in concentrations between 100 and 0.04 mg/mL (W), between 75 and 0.035 mg/mL (ET and EA), and between 3 and 0.0012 mg/mL (H). The plates were incubated at 36°C for 24 hours. After turbidity was observed, we followed the same assessment standards used for the essential oil.

Determination of the Minimum Bactericidal Concentration (MBC)

The MBC was determined based on the methodology described by Santurio et al. (2007). From the wells in which there was no visible bacterial growth in the MIC test, prior to the addition of TTC, we withdrew an aliquot of 10 µL and inoculated it on the Mueller-Hinton agar surface. The plates were incubated for 24 hours at 36°C and, after this procedure, the MBC was defined as the lowest concentration of the extract/oil able to cause the death of the inoculum. The tests of MIC and MBC were carried out in triplicate.

Distilled water, ethanol and ethyl acetate were used as negative control; gentamicin was used as positive control for bacteria; and nystatin was used for *C. albicans* (Table 1). Synthetic antimicrobials were tested at concentrations of 100 to 0.78 mg/mL.

Antioxidant activity

The measurement of the activity of free radicals scavenging (2.2-diphenyl-1-picrylhydrazyl, DPPH) was assessed as described by Scherer and Godoy (2009) and Rufino et al. (2007) with modifications. For the analysis, 0.1 mL of each dilution of samples or patterns were placed in

test tubes containing 3.9 mL DPPH radical (0.2 mM) diluted with methanol and homogenized in a test tube agitator. For the negative control, we used 0.1 mL control solution (methyl alcohol, acetone and water) with 3.9 mL DPPH radical, which were homogenized. We used the commercial synthetic antioxidant butylated hydroxytoluene (BHT) following the same procedure used for the negative control. Methyl alcohol was used as whitening agent in order to calibrate the spectrophotometer (UV mini-1240, Shimadzu Co., Japan). The mixtures were incubated in the absence of light at room temperature until measurement. Subsequently, the absorbance at 515 nm was measured using a spectrophotometer and monitored every 30 minutes until stabilization. The tests were carried out in triplicate.

The DPPH index was calculated using the antioxidant activity equation (%) = [(Abs0 -Abs1) /Abs0] × 100, where Abs0 is the absorbance of the whitening agent and Abs1 the absorbance of the sample.

The concentrations of the samples (extracts and EO) responsible for 50% decrease in the initial activity of DPPH free radical (IC_{50}) were calculated through linear regression of the antioxidant activity.

Statistical analysis

The data obtained by calculating the DPPH index and IC_{50} were analyzed through Tukey test at 5% significance using the Sisvar software (Ferreira, 2007).

RESULTS AND DISCUSSION

The tests conducted for phytochemical screening (Table 2) showed that the aqueous extract had only the classes tannins and flavonoids. The ethanolic extract showed the greatest number of classes of substances: tannins; saponins; flavonoids; and terpenes. The extract with ethyl acetate solvent only showed flavonoids and the hexanic extract did not show positive results for the classes of substances tested.

It is known that the chemical constitution of Rosaceae includes especially tannins (Okuda et al., 1992), flavonoids (Harbone, 1998), triterpenes, and steroids (Wallaart, 1980). The data obtained in our research agree with studies of these authors, except for the class of steroids, which was not found in any of the extracts tested.

Three compounds were found in the volatile composition of essential oil from *P. myrtifolia*, and the largest class of compounds identified belonged to aldehydes, represented by benzaldehyde, which constituted approximately 97% of the total area of the chromatogram peaks. It was followed by lower percentages of alcohol classes (3-hexen-1-ol) and esters (benzyl benzoate), with 0.07 and 0.09% total peak area, respectively (Table 3). These data agree with those found by Ibarra-Alvarado et al. (2009), when they identified the volatile compounds of oil from *P. Serotina*, they also detected benzaldehyde as majoritary compound. It is known that benzaldehyde is one of the main components responsible for the characteristic odor of essential oils (Kerdogan-Orhan and Kartal, 2011) and it is related to various biological activities, such as antimicrobial and antifungal (Fujii et al., 2005).

The results summarized in Table 4 indicate that all extracts and the essential oil tested showed antimicrobial activity against the microorganisms assessed, with exception of the hexanic extract that showed no activity.

The essential oil had MIC values ranging from 3500 to 1750 µg/mL over the microorganisms tested. For the majority of microorganisms, the MBC was 7000 µg/mL, and 3500 µg/mL only for *P. aeruginosa* and *S. Typhimurium*. The activity found in the oil can be due to the presence of benzaldehyde in its composition. This compound is environmentally safe when used as an antimicrobial, considering its wide spectrum of inhibitory effect. It is also used as a bactericide and fungicide. Benzaldehyde activity has similarities to the antimicrobial activity of phenols, because it interacts with the surface of the cell and leads to cell death by disintegration of the cell membrane and release of intracellular components (Alamri et al., 2012).

Aqueous, ethanolic and ethyl acetate extracts had MIC values ranging from 0.04 to 150 mg/mL, comparable with standard antimicrobials, which ranged from 3.125 to 6.25 mg/mL. Thus, the extracts were as potent antimicrobials inhibiting the growth of microorganisms' strains as synthetic antimicrobials. With respect to gram-positive microorganisms, the same extracts had smaller MIC (0.04 to 4.69 mg/mL) compared with gentamicin (6.25 mg/mL). Regarding ethanolic extracts, *C. albicans* also had lower MIC value (4.69 mg/mL) compared to nystatin (6.25 mg/mL). When the different plant extracts (aqueous, ethanolic and ethyl acetate), were assessed regarding the gram-negative microorganisms, they had MIC ranging from 9.38 to 150 mg/mL, which were higher concentrations when compared to gentamicin concentrations (3.125 to 6.25 mg/mL). The same ratio found in the MIC was observed with respect to MBC, with values ranging from 0.09 to 150 mg/mL.

A growing number of mechanisms with inhibitory action-such as the secondary metabolites-have been assigned to active compounds present in plant extracts. Thus, the antimicrobial activity observed in aqueous, ethanolic and ethyl acetate extracts can be related to the presence of flavonoids (W, ET, and EA), tannins (ET and W), triterpenoids (ET), and saponins (ET) (Table 2), which have already proved active in different studies described in the literature (Recio et al., 1989).

It is known that the presence of flavonoids is related to most antimicrobial activities of extracts, including antibacterial (Gibbons, 2008) and antifungal potential (Cao et al., 2008). In this study, we observed greater activity against gram-positive bacteria. This fact can result from the presence of flavonoids, agreeing with the results found by Taleb-Contini et al. (2003).

The compounds commonly related to antimicrobial activity, such as flavonoids, tannins, saponins, and triterpenes, generally act in the microorganism's membrane or cell wall. Flavonoids act in the bacterial cell through complexes between proteins and the cell wall causing its breakage (Taguri et al., 2004). Tannins act in microorganisms by preventing their

growth through the inhibition of nutrients transport to the cell caused by the formation of complexes between the organism and the cell wall (McSweeney et al., 2001). The action mechanism of triterpenes in microorganisms is related to the breakage of lipophilic compounds of microbial membranes (Bagamboula et al., 2004). Lastly, with respect to the saponins, they act actively in the membrane sterols (Sparg et al., 2004).

The difference between the activity found in the extracts can be attributed to the fact that the components extracted from aromatic plants with antimicrobial activity have greater solubility in solvents like ethanol, compared to hexane, for example (Cowan, 1999). Similarly, the results obtained agree with those found by Rojas et al. (2006) in which the ethanolic extract has antimicrobial activity in comparison with hexane extract, confirming the fact that the latter did not have activity at the concentration tested.

In general, aqueous and ethanolic extracts demonstrated inhibitory activity regarding all strains tested in smaller concentrations when compared to ethyl acetate extract, agreeing with Yiğit et al. (2009), who reported antimicrobial activity for ethanolic and aqueous extracts from *P. armeniaca* against gram-negative and gram-positive bacteria and yeast as *C. albicans*.

With respect to antioxidant activity, it should be noted that the IC_{50} values are inversely related to the percentage of DPPH sequestration, since the higher the rate of sequestration, the lower IC_{50}, establishing a relationship between the values (Table 5).

The results of the antioxidant activity, expressed as IC_{50}, showed no significant difference between the synthetic antioxidant (BHT) and aqueous, ethanolic and ethyl acetate extracts; thus, they can be considered excellent antioxidants. On the other hand, there was significant difference ($p<0.5$) when compared to BHT, essential oil and hexanic extract, and no antioxidant activity was detected in these compounds. The same correlation can be observed in relation to the DPPH sequestration percentage. It is worth mentioning that the IC_{50} determines the minimum sample amount needed to reduce the DPPH free radical absorbance

by 50%. However, the analysis of antioxidant activity expressed in percentages can underestimate the real potential of the samples.

According to Gao et al. (1999) phenolic compounds such as flavonoids, triterpenes and tannins are excellent antioxidants. These compounds were found in the phytochemical screening of the extracts tested (Table 2). Ethno-pharmacological data have been reported in studies conducted on the genus Prunus regarding the relationship of antioxidant activity and the presence of flavonoids (Nakatani et al., 2000). The values obtained for the DPPH sequestration index-which are similar to those obtained for BHT, aqueous and ethanolic extracts-agree with the data found by Yiğit et al. (2009).

The non-detection of antioxidant activity with respect to the essential oil may be due to the presence of its majoritary compound, i.e., benzaldehyde, which features moderate to low antioxidant activity (Thanh and Hoai, 2012).

The genus *Prunus* has economic importance for the food and phytopharmaceutical industries. The literature reports more than 100 patents involving different *Prunus* species in their formulation for multiple purposes: skin whitening (Pieroni et al., 2004); sunscreens and anti-aging skin care (Sachdeva and Katyal, 2011); essential oils used in the chemical industry (Bachheti et al., 2012); livestock food (Khanal and Subba, 2001); antimalarial treatment (Muñoz et al., 2000); asthma treatment (Karani et al., 2013); and cardiovascular disease prevention (Negishi et al., 2007).

The increased growth of antimicrobial-resistant microorganisms commonly used is one of the most serious threats to the successful treatment of microbial diseases. Thus, the search for products that replace synthetic antimicrobials, such as essential oils and plant extracts, is increasing primarily because they are associated with the treatment of infectious diseases (Bharathi et al., 2010). Therefore, testing new natural compounds with antimicrobial action is of great value.

Within this context, it is worth mentioning the importance of phytochemical studies, since they confirm the biological activities found. It is also worth noting the importance of preliminary studies to determine the activity of these compounds so that they can serve as the basis for subsequent studies in order to isolate different compounds with antimicrobial activity. The antioxidant activity has to be determined, since the compound has to be both antimicrobial and antioxidant.

In conclusion, the presence of flavonoids and terpenoids, among other metabolites, was detected in aqueous, ethanolic and ethyl acetate extracts. With respect to the essential oil, benzaldehyde was found as the majoritary compound. Regarding antimicrobial activity, microorganisms proved susceptible to aqueous, ethanolic and ethyl acetate extracts, and essential oil, demonstrating the antimicrobial potential of *P. myrtifolia*. With respect to antioxidant activity, the ethanolic, aqueous and ethyl acetate extracts had significant values comparable to those of synthetic antioxidant.

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Table 1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of distilled water, organic solvents and reference antibiotics on pathogenic microorganisms.

Microorganisms	MIC/MBC (mg/mL)				
	Distilled water	Ethanol	Ethyl acetate	Gentamycin	Nystatin
P. aeruginosa ATCC 27853	Na	Na	Na	6,25/6,25	Nt
S. Typhimurium ATCC 14028	Na	Na	Na	3,125/6,25	Nt
P. mirabilis ATCC 25933	Na	Na	Na	6,25/6,25	Nt
K. pneumoniae ATCC 13883	Na	Na	Na	6,25/6,25	Nt
E. coli ATCC 25922	Na	Na	Na	6,25/6,25	Nt
E. faecalis ATCC 19433	Na	Na	Na	3,125/6,25	Nt
S. epidermidis ATCC 12228	Na	Na	Na	6,25/6,25	Nt
S. aureus ATCC 25923	Na	Na	Na	6,25/6,25	Nt
B. subtillis CCD-04	Na	Na	Na	6,25/6,25	Nt
C. albicans ATCC 10231	Na	Na	Na	Nt	6,25/6,25

* Na: No activity (100<); Nt: Not tested

Table 2. Classes of secondary metabolites identified in different extracts from *Prunus myrtifolia*.

Classes of metabolites	EXTRACTS			
	W	ET	EA	H
Tannins	+	+	-	-
Alkaloids	-	-	-	-
Coumarins	-	-	-	-
Saponins	-	+	-	-
Anthocyanins	-	-	-	-
Anthocyanidins	-	-	-	-
Flavonoids	+	+	+	-
Triterpenoids	-	+	-	-
Steroids	-	-	-	-

*- = absent; + = present; W = aqueous extract; ET = ethanolic extract; EA = ethyl acetate extract; H = hexane extract.

Table 3. Volatile composition of *Prunus myrtifolia* through GC-MS

RT	Compound name	KI	Area (%)
5,74	3-Hexen-1-ol	852	0,07
10,22	Benzaldehyde	964	96,96
57,22	Benzyl benzoate	1759	0,09

* RT: Retention time; KI: Kováts retention index calculate.

Table 4. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of essential oil and different extracts of Prunus myrtifolia on pathogenic microorganisms.

Microorganisms	MIC/MBC			
	EO (μg/mL)	W (mg/mL)	ET (mg/mL)	EA (mg/mL)
P. aeruginosa ATCC 27853	3500/3500	12,5/12,5	9,38/18,75	37,5/75
S. Typhimurium ATCC 14028	1750/3500	12,5/25	18,75/37,5	150/150
P. mirabilis ATCC 25933	3500/7000	12,5/12,5	18,75/18,75	37,5/75
K. pneumoniae ATCC 13883	3500/7000	12,5/12,5	18,75/37,5	37,5/37,5
E. coli ATCC 25922	1750/7000	12,5/25	9,38/37,5	37,5/75
E. faecalis ATCC 19433	1750/7000	12,5/25	9,38/18,75	9,38/18,75
S. epidermidis ATCC 12228	3500/7000	1,56/1,56	1,18/2,35	4,69/9,38
S. aureus ATCC 25923	3500/7000	0,04/0,09	0,07/0,15	2,34/4,68
B. subtillis CCD-04	3500/7000	3,13/6,25	4,69/4,69	4,69/9,38
C. albicans ATCC 10231	3500/7000	6,25/6,25	4,69/9,37	9,38/9,38

* EO: essential oil; W: Aquous extract; ET: Ethanolic extract; EA: Ethyl acetate extract. Hexane extract – No activity.

494 **Table 5.** DPPH average and standard deviation (% sequestration) and IC_{50} values of essential
495 oil and different extracts from *Prunus myrtifolia* in the different concentrations tested.

Extracts/Oil	Antioxidant activity (%)	IC_{50} (mg/mL)
BHT	95.85±0.07[a]	11.52±0.96 [a]
W	91.27±0.67[a]	20.12±0.05 [a]
ET	94.12±0.64[a]	15.43±0.01 [a]
EA	78.49±0.98[a]	14.58±0.28 [a]
H	2.81±0.039[b]	186.26±0.01[b]
EO	8.69±0.97[b]	175.17±0.99 [b]

496 *Standard error followed by the same letter in the column do not differ trough Tukey test
497 ($p<0.05$); EO = Essential oil; W = Aqueous extract; ET = Ethanolic extract; EA = Ethyl
498 acetate extract; H = Hexane extract.

CAPÍTULO 2:

Composição química, atividade antioxidante e antimicrobiana de extratos vegetais de seis espécies vegetais frente a sorotipos de *Salmonella* de origem aviária.

O artigo segue as normas sugeridas pela revista "Revista Brasileira de Plantas Medicinais" citada em Anexos Capítulo 2

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Composição química, atividade antioxidante e antimicrobiana de extratos vegetais de seis espécies vegetais frente a sorotipos de *Salmonella* de origem aviária.

WEBER, L.D.[1], PINTO, F.G.S.[1], BONA, E.M.[2], SCUR, M.C.[1], TEMPONI, L.G.[1], JORGE, T.C.M.[1], ALVES, L.F.A.[1]

[1]Universidade Estadual do Oeste do Paraná (UNIOESTE), Centro de Ciências Biológicas e da Saúde, UNIOESTE/CCBS, Laboratório de Biotecnologia Agrícola, Campus Cascavel, Rua Universitária, 1.619, Caixa Postal 701, Cascavel, PR, CEP: 85819-110, e-mail: layweber@gmail.com – Laís Dayane Weber.

RESUMO

Avaliou-se o perfil fitoquímico, ação antioxidante e antimicrobiana dos extratos vegetais etanólico e aquoso de seis plantas brasileiras obtidos das folhas secas de *Maytenus aquifolia* Mart. (espinheira-santa), *Plinia cauliflora* (Mart.) O. Berg (jabuticabeira), *Ocotea spixiana* (Nees) Mez. (canela-branca), *Psidium guajava* L. (goiabeira), e *Ricinus communis* L. (mamona) e *Schinus molle* L. (aroeira). A atividade antimicrobiana *in vitro* dos extratos vegetais foi testada frente a trinta e seis sorotipos de *Salmonella* de origem avícola pelo método de microdiluição em caldo com a determinação da Concentração Inibitória Mínima (CIM) e a Concentração Bactericida Mínima (CBM). A ação antioxidante dos mesmos foi avaliada pelo método de DPPH (2,2-difenil-1-picril-hidrazila). O perfil fitoquímico detectou componentes com potencial antimicrobiano e antioxidante em todos os extratos, assim como um percentual de captura do DPPH superior a 65%, demonstrando o elevado potencial antioxidante dos extratos testados. Nos testes de microdiluição em caldo, observou-se a atividade antimicrobiana de todos os extratos testados, sendo que em geral os extratos etanólicos foram mais eficazes quando comparados aos aquosos, sendo o extrato etanólico de *P. cauliflora* seguido por *P. guajava* de maior efeito bacteriostático. As CIMs variaram entre 1,56-100 mg.mL^{-1} e a CBM entre 3,13-100 mg.mL^{-1}. Esses resultados confirmaram o potencial antimicrobiano e antioxidante desses extratos vegetais.

Palavras-chave: Microdiluição, bactericida, bacteriostático, DPPH.

ABSTRACT: Antimicrobial activity of extracts from plants native to Brazil control of *Salmonella* as avian origin. It was evaluated the phytochemical profile , antioxidant and antimicrobial activity of ethanolic and aqueous plant extracts from six Brazilian plants obtained

from the dried leaves of *Maytenus aquifolia* Mart., *Plinia cauliflora* (Mart.) O. Berg, *Ocotea spixiana* (Nees) Mez., *Psidium guajava* L., *Ricinus communis* L. and *Schinus molle* L.. The in vitro antimicrobial activity of plant extracts was tested against thirty-six serotypes of Salmonella from poultry products by the broth microdilution method to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The antioxidant properties of these was evaluated by DPPH (2,2- diphenyl-1-picryl-hidrazila) method. The phytochemical profile detected components with antimicrobial and antioxidant potential in all extracts , as a percentage capture of DPPH than 65% , demonstrating the high antioxidant activity of the tested extracts. In microdilution tests, we observed the antimicrobial activity of all tested extracts, and in general the ethanol extracts were more effective when compared to aqueous and ethanol extract of *P. cauliflora* followed by *P. guajava* higher end bacteriostatic. The MIC ranged from 1.56 to 100 $mg.mL^{-1}$ and MBC of 3.13 to 100 $mg.mL^{-1}$. These results confirmed the antioxidant and antimicrobial potential of these plant extracts.

Key Word: Antimicrobial, MIC, MBC, DPPH.

INTRODUÇÃO

O gênero *Salmonella* é constituido por patógenos de importancia internacional, de difícil controle e comumente encontrada na cadeia avícola e consequentemente na carne de frango, podendo causar surtos de intoxicação alimentar em humanos e prejuízos econômicos no setor (SHINOHARA et al., 2008).

Fatores que contribuem para a patogenicidade de *Salmonella* spp. são seu grande número de sorotipos, a capacidade de adaptação a vários hospedeiros e a predisposição de adquirir e transmitir alelos de resistência à antimicrobianos (EUROPEAN FOOD SAFETY AUTHORITY, 2008a) sendo este último em destaque, uma devido ao uso intensivo de agentes antimicrobianos são mais frequentes o surgimento de cepas multiresistentes (EUROPEAN FOOD SAFETY AUTHORITY, 2008b).

O uso de antimicrobianos como promotores de crescimento em frangos foi abolina pela união européia, por isso, além da substituição desses produtos se dar pela tentativa de impedir o surgimento de micro-organismos resistentes, os países exportadores também precisam se adequar as leis de mercado, fazendo-se necessária a busca por proutos alternativos ao uso dos antimicrobianos sintéticos (PcMulin, 2004).

Com isso, a utilização de produtos naturais como potencial agente antimicrobiano chama a atenção das indústrias na busca de novos compostos que não agridam o meio ambiente (MESA-ARANGO et al., 2009). Neste contexto, os extratos vegetais vêm ganhando espaço nas pesquisas para o controle de diferentes micro-organismos patogênicos e, por isso, tem-se buscado novas plantas, a fim de que os extratos sejam considerados como um produto

sanitário alternativo, seguro e saudável quando comparado aos antimicrobianos sintéticos (LOVATTO et al., 2012). Contudo, grande parte das pesquisas realizadas mencionam testes de extratos frente aos micro-organismos referência, sendo necessário o estabelecimento de parâmetros mais precisos quanto ao real potencial antimicrobiano de extratos em diferentes sorotipos de *Salmonella*, uma vez que se tem relatado diversos sorotipos como responsáveis por casos e surtos de salmonelose humana no Brasil e no exterior, muitos deles envolvendo alimentos de origem avícola (KOTTWITZ et al. 2008).

No intuito de investigar plantas nativas do Brasil com potencial antioxidante e antimicrobiano, o presente estudo realizou o rastreamento fitoquímico de metabolitos secundários e potencial antioxidante de extratos vegetais etanolicos e aquosos obtidos das folhas de *Maytenus aquifolia* Mart. (espinheira-santa), *Plinia cauliflora* (Mart.) O. Berg (jabuticabeira), *Ocotea spixiana* (Nees) Mez. (canela-branca), *Psidium guajava* L. (goiabeira), e *Ricinus communis* L. (mamona) e *Schinus molle* L. (aroeira) bem como sua atividade antimicrobiana frente a diferentes sorotipos de *Salmonella* de origem avícola.

MATERIAL E MÉTODOS

Plantas utilizadas

As folhas de *Maytenus aquifolia* Mart. (espinheira-santa) (Celastraceae), *Plinia cauliflora* (Mart.) O. Berg (jabuticabeira) (Myrtaceae), *Ocotea spixiana* (Nees) Mez. (canela-branca) (Lauraceae), *Psidium guajava* L. (goiabeira) (Myrtaceae), *Ricinus communis* L. (mamona) (Euphorbiaceae) e *Schinus molle* L. (aroeira) (Anarcadiaceae) foram coletadas no período da manhã na região Oeste do Paraná, Brasil de janeiro a maio de 2012. O material foi identificado pela Profª Drª Lívia Godinho Temponi e incorporado no Herbário da Universidade Estadual do Oeste do Paraná (UNOP), sob o número de voucher 6899, 6882, 6882, 6882, 6882 e 6882, respectivamente.

Preparo dos extratos

Os extratos aquosos e etanolicos foram obtidos segundo a metodologia de Bona et al. (2012). As folhas coletadas foram secas a 40 ºC e moídas em moinho de facas. Para a realização dos extratos, adicionou-se ao material vegetal triturado álcool etílico P.A. ou água destilada estéril na proporção de 2:10 (p/v) para maceração por 24 h em agitador rotativo a 23 ºC. Para o extrato etanólico, a concentração realizou-se em evaporador rotativo a 40 ºC, diluído com água destilada estéril na concentração de 200 $mg.mL^{-1}$ e ambos filtrados em papel de filtro. Para ambos os extratos, uma última filtração a vácuo foi realizada utilizando uma membrana filtrante com porosidade de 0,45mm. As soluções foram armazenadas à 4 °C.

Rastreamento fitoquímica

Os principais metabólitos secundários foram detectados de acordo com metodologia desenvolvida por Matos (1997). Dessa forma, utilizou-se o teste de fenóis e taninos, antocianinas, antocianididas e flavonoides, flavonóis, flavanonas, favanonois e xantonas, catequinas, esteróis e triterpenóis, saponinas e alcaloides.

Atividade antioxidante

A medição da atividade de sequestro de radicais livres DPPH (2,2-difenil-1-picril-hidrazil) foi avaliada como descrito por Scherer e Godoy (2009). Para a análise, 0,1 mL de cada amostra ou padrões foram adicionados em tubos de ensaio que continham 3,9 mL do radical DPPH (0,2 mM). Para o controle negativo foi utilizado 0,1 mL de solução controle (álcool metílico, acetona e água), como padrão utilizou-se o antioxidante sintético comercial BHT (butil hidroxi tolueno) e como branco foi utilizado álcool metílico, a fim de calibrar o espectrofotômetro (UV mini-1240, Shimadzu Co.). As misturas foram incubadas na ausência de luz à temperatura ambiente até medição, utilizando-se um espectrofotômetro a 515 nm até a estabilização dos valores. Após, os dados foram analisados calculando-se o índice DPPH e EC50 e os analisando pelo teste de Tukey, utilizando-se o programa Sisvar (Ferreira, 2007).

Micro-organismos

Cento e dezoito amostras de *Salmonella* provenientes de frango de corte de diferentes aviários da região Oeste do Paraná foram obtidas em um Laboratório de Sanidade Avícola no Paraná, credenciado pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA), durante o período de 2006 a 2010. A sorotipagem foi realizada pelo Instituto Adolfo Lutz, São Paulo, Brasil. As amostras coletadas estavam distribuídas em trinta e seis sorotipos, e um representante de cada sorotipo foi selecionado aleatoriamente para avaliar a suscetibilidade aos extratos vegetais. Como cepa referência, utilizou-se a *Salmonella* Typhimurium ATCC 14028 (American Type Culture Collection).

Teste de microdiluição em caldo

As suspensões bacterianas foram diluídas a fim de se obter um inóculo na concentração de 1×10^5 UFC.mL^{-1}. A Concentração inibitória mínima (CIM) dos extratos foi determinada pela técnica da microdiluição em caldo proposta por Ayres et al. (2008). Alíquotas (15 µL) da diluição foram distribuídas em placas de 96 poços de microtitulação contendo 150 µL de caldo Mueller Hinton (MH) (concentração dupla), com a adição anterior dos extratos. Os extratos foram diluídos em concentrações entre 0,04 e 100 mg.mL^{-1}. As placas foram incubadas a 36 °C por 24 h. Após avaliação visual dos resultados, cada poço recebeu uma alíquota de 10 µL de cloreto trifenil de tetrazólio (CTT) a 0,5%, re-incubaram por 3h a 36 °C. A CIM foi definida como a menor concentração do extrato em mg.mL^{-1} capaz de impedir o crescimento microbiano

(SARTORATTO et al., 2004). A partir dos poços onde não houve crescimento bacteriano visível no teste da CIM, anterior a adição de CTT, foi retirada uma alíquota de 10 µL e inoculada na superfície do ágar MH. As placas foram incubadas por 24h a 36 ºC e após foi definida a Concentração bactericida mínima (CBM) como a menor concentração do extrato capaz de causar a morte do inóculo (Santútio et al., 2007). Os ensaios de CIM e CBM foram realizados em triplicata.

RESULTADOS E DISCUSSÃO

O presente estudo demonstrou que os extratos etanólicos e aquosos das folhas das espécies avaliadas apresentavam metabólitos secundários (Tabela 1) em sua maioria com potencial antimicrobiano reportado. Segundo Pinho et al. (2012) embora as folhas apresentam menor concentração de agentes antimicrobianos, a elaboração de extratos através delas apresenta como vantagem promover uma prática sustentável à sobrevivência da planta. Sendo assim, observou-se por meio do rastreamento fitoquímico a presença das mesmas classes de metabólitos secundários para todos os extratos das plantas, sendo eles: taninos, flavonoides e triterpenoides, com exceção dos extratos de *P. cauliflora* e *P. guajava* que apresentaram também as cumarinas e *O. spixiana* e *M. aquifolia* que apresentam alcalóides além dos metabólitos citados anteriormente (Tabela 1), compostos estes que apresentam atividade antimicrobiana relatada.

TABELA 1. Resultados da triagem fitoquímica realizada com os extratos etanólicos (Et) e aquosos (Aq) de *Plinia cauliflora* (Pc), *Schinus molle* (Sc), *Ricinus communis* (Rc), *Psidium guajava* (Pg); *Ocotea spixiana* (Os) e *Maytenus aquifolium* (Ma).

Classes de metabólitos	EXTRATOS											
	Pc		Sm		Rc		Pg		Os		Ma	
	Et	Aq	Et	Aq	Et	Aq	Et	Aq	Et	Aq	Et	Aq
Taninos	+	+	+	+	+	+	+	+	+	+	+	+
Alcaloides	-	-	-	-	-	-	-	-	+	-	+	+
Cumarinas	+	+	-	-	-	-	+	+	-	-	-	-
Saponinas	-	-	-	-	-	-	-	-	-	-	-	-
Antocianinas	-	-	-	-	-	-	-	-	-	-	-	-
Antocianidinas	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoides	+	+	+	+	+	+	+	+	+	+	+	+
Triterpenoides	+	+	+	+	+	+	+	+	+	+	+	+
Esteroides	-	-	-	-	-	-	-	-	-	-	-	-

+ = presente; -= ausente.

Quanto ao potencial antioxidante (Tabela 2), todos os extratos apresentaram percentual de sequestro superior a 65%, sendo que os extratos etanólico de *M. aquifolia* (93,30%), *P. guajava* (93,46%) e aquoso de *O. spixiana* (93,60%) foram os melhores, seguido do extrato aquoso de *M. aquifolia* (92,04%) e etanólico de *P. cauliflora* (92,54%), e, embora estes extratos

tenham apresentado altos valores de sequestro de radicais livres, os valores foram significativamente diferentes do BHT (antioxidante sintético). Quanto aos valores de IC50, a relação estatística entre os dados permaneceu a mesma, uma vez que o % de sequestro e o IC50 são inversamente proporcionais, ou seja, quanto maior o valor de % de sequestro menor o IC50. Segundo Gao et al., (1999), os compostos fenólicos, como flavonoides, triterpenos e taninos são excelentes antioxidantes, compostos esses que foram encontrados na triagem fitoquímica dos extratos testados (NAKATANI et al., 2000). A variação da resposta da atividade antioxidante pode ser devido à concentração de metabólitos presente em cada extrato.

TABELA 2. Atividade antioxidante (expresso pela % de sequestro e IC_{50}) dos extratos vegetais etanolicos e aquosos de *Plinia cauliflora, Schinus molle, Ricinus communis, Psidium guajava, Ocotea spixiana e Maytenus aquifolia.*

Extratos	% de sequestro	IC50
BHT	95,84 ± 0,14^{a}	9,24 ± 2,79^{a}
P. cauliflora EE	92,54 ± 0,14^{c}	15,18 ± 2,79^{b}
P. cauliflora EA	79,27 ± 0,14^{f}	40,36 ± 2,79^{cd}
S. molle EE	84,28 ± 0,14^{e}	40,96 ± 2,79^{c}
S. molle EA	79,37 ± 0,14^{d}	42,05 ± 2,79^{c}
R. communis EE	79,74 ± 0,14^{f}	40,26 ± 2,79^{c}
R. communis EA	67,03 ± 0,14^{h}	63,36 ± 2,79^{e}
P. guajava EE	93,46 ± 0,14^{b}	13,72 ± 2,79^{b}
P. guajava EA	89,28 ± 0,14^{d}	21,57 ± 2,79^{b}
O. spixiana EE	70,79 ± 0,14^{g}	56,30 ± 2,79^{de}
O. spixiana EA	93,30 ± 0,14^{b}	14,02 ± 2,79^{b}
M. aquifolia EE	93,60 ± 0,14^{b}	13,45 ± 2,79^{b}
M. aquifolia EA	92,04 ± 0,14^{c}	16,39 ± 2,79^{b}

BHT: antioxidante sintético. Média ± erro padrão seguido pela mesma letra na coluna não diferem entre si pelo teste de Tukey $p<0,05$.

O teste de microdiluição em caldo indiciou a atividade antimicrobiana dos extratos vegetais etanolicos e aquosos (Tabela 3 e 4). Os extratos etanólicos testados (*M. aquifolia*, *P. cauliflora*, *O. spixiana*, *P. guajava*, *R. communis* e *S. molle*) apresentaram CIM variando de 1,56 a 100 $mg.mL^{-1}$ frente a todos os sorotipos testados, e CBM variando de 3,13 a 100 $mg.mL^{-1}$. Entretanto, não foram detectados valores de CBM relacionados ao extrato etanólico *S. molle* frente a todos os sorotipos testados e para os extratos etanolicos de *R. communis, O. spixiana* e *P. cauliflora* mais de 50% dos sorotipos não apresentaram valores CBM nas concentrações testadas (Tabela 2). Em relação aos extratos aquosos observou-se valores de CIM variando de 12,5 a 100 $mg.mL^{-1}$ frente a todos os sorotipos testados, exceto para os extratos *R. communis* e *O. spixiana* no qual não apresentaram atividade antimicrobiana nas concentrações testadas frente aos diferentes sorotipos. O extrato aquoso *P. cauliflora* apresentou valores de CBM

variando de 12,5 a 50 mg.mL^{-1}, contudo, os extratos *S. molle* e *M. aquifolia* não apresentaram valores CBM frente a todos os sorotipos testados e o extrato aquoso *P. guajava* apresentou valores de CBM para mais de 50% dos sorotipos testados.

TABELA 3. Concentração inibitória mínima **(**CIM) e Concentração bactericida mínima (CBM) de extratos vegetais etanólicos frente à sorotipos de *Salmonella,* isolados de aviários no Oeste do Paraná.

	EXTRATO ETANÓLICO CIM/CBM (mg.mL^{-1})					
SOROTIPOS	Pc	Sm	Rc	Pg	Os	Ma
Morehead	3,13/6,25	50/>LD	12,5/100	6,25/12,5	25/50	12,5/100
Lexington	3,13/6,25	100/>LD	25/>LD	6,25/12,5	25/>LD	12,5/100
Give	1,56/6,25	100/>LD	25/>LD	6,25/12,5	25/100	12,5/100
Panamá	3,13/6,25	50/>LD	25/>LD	6,25/12,5	25/100	12,5/100
Typhimurium	1,56/3,13	100/>LD	25/>LD	6,25/12,5	25/>LD	12,5/100
Rissen	3,13/6,25	100/>LD	50/>LD	6,25/12,5	25/50	12,5/100
Albany	3,13/6,25	100/>LD	100/100	3,13/3,13	25/50	12,5/100
Gallinarum	1,56/6,25	100/>LD	100/100	6,25/6,25	25/>LD	12,5/100
Cerro	3,13/6,25	100/>LD	12,5/100	6,25/6,25	25/>LD	12,5/100
Infantis	3,13/3,13	100/>LD	12,5/50	6,25/12,5	25/>LD	12,5/100
Schwarzengrund	3,13/6,25	100/>LD	12,5/100	6,25/6,25	25/>LD	12,5/>LD
Worthington	3,13/6,25	100/>LD	12,5/100	3,13/12,5	25/>LD	12,5/>LD
Saintpaul	3,13/6,25	100/>LD	12,5/100	3,13/12,5	25/>LD	12,5/100
Braenderup	3,13/6,25	100/>LD	12,5/100	3,13/12,5	25/>LD	12,5/100
Montevideo	3,13/6,25	100/>LD	12,5/100	3,13/12,5	25/>LD	12,5/100
Mbandaka	3,13/6,25	100/>LD	12,5/100	3,13/6,25	25/>LD	12,5/>LD
Ohio	1,56/3,13	100/>LD	25/25	3,13/12,5	25/50	12,5/>LD
Agona	1,56/3,13	100/>LD	25/25	6,25/12,5	25/50	12,5/>LD
Senftenberg	1,56/6,25	100/>LD	25/>LD	6,25/12,5	50/100	12,5/>LD
Corvallis	1,56/3,13	100/>LD	25/>LD	3,13/6,25	25/>LD	6,25/12,5
Hadar	1,56/3,13	100/>LD	50/>LD	3,13/6,25	25/>LD	12,5/>LD
Grumpensis	1,56/3,13	100/>LD	25/>LD	3,13/6,25	25/>LD	12,5/>LD
Gafsa	1,56/6,25	100/>LD	25/>LD	3,13/12,5	25/>LD	12,5/>LD
Orion	1,56/3,13	100/>LD	25/>LD	3,13/6,25	25/>LD	12,5/>LD
Tennessee	3,13/6,25	100/>LD	25/100	6,25/25	25/50	12,5/>LD
Cubana	1,56/3,13	100/>LD	25/>LD	6,25/6,12	25/>LD	25/>LD
Kentucky	1,56/3,13	100/>LD	12,5/>LD	6,25/12,5	25/50	25/>LD
Bareilly	3,13/6,25	100/>LD	25/>LD	3,13/12,5	25/50	25/>LD
Livingstone	3,13/6,25	100/>LD	25/>LD	6,25/12,5	25/50	25/>LD
Minnesota	3,13/6,25	100/>LD	12,5/100	6,25/12,5	25/50	12,5/>LD
Branderburg	3,13/6,25	100/>LD	12,5/100	6,25/12,5	25/50	12,5/>LD
Enteritidis	3,13/6,25	100/>LD	12,5/25	6,25/12,5	25/>LD	12,5/>LD
Newport	3,13/6,25	100/>LD	12,5/>LD	6,25/12,5	25/>LD	12,5/>LD
Entérica	3,13/6,25	100/>LD	12,5/>LD	6,25/12,5	25/50	12,5/>LD
Derby	1,56/3,13	100/>LD	12,5/>LD	6,25/12,5	25/50	12,5/>LD
Heidelberg	3,13/3,13	100/>LD	12,5/>LD	6,25/12,5	2550	12,5/>LD
Typhimurium ATCC	3,13/6,25	100/>LD	12,5/>LD	6,25/12,5	25/100	12,5/>LD

AMP: Ampicilina; CEF: Cefalotina; GEN: Gentamicina; NAL: Ácido Nalidixico; TET: Tetraciclina; >LD: Maior que o limite de detecção; Pc: *Plinia cauliflora*; Sm: *Schinus molle*; Rc: *Ricinus communis*; Pg: *Psidium guajava*; Os: *Ocotea spixian*a; Ma: *Maytenus aquifolium*.

TABELA 4. Concentração inibitória mínima (CIM) e Concentração bactericida mínima (CBM) de extratos vegetais aquosos frente à sorotipos de *Salmonella,* isolados de aviários no Oeste do Paraná.

	EXTRATO AQUOSO CIM/CBM ($mg.mL^{-1}$)			
SOROTIPOS	Pc	Sm	Pg	Ma
Morehead	12,5/12,5	100/>LD	50/50	50/>LD
Lexington	25/25	100/>LD	100/>LD	50/>LD
Give	12,5/50	100/>LD	100/>LD	50/>LD
Panamá	25/50	100/>LD	100/>LD	100/>LD
Typhimurium	25/25	100/>LD	100/>LD	50/>LD
Rissen	25/50	100/>LD	100/>LD	50/>LD
Albany	12,5/12,5	100/>LD	50/50	50/>LD
Gallinarum	50/50	100/>LD	50/100	50/>LD
Cerro	12,5/12,5	100/>LD	100/100	50/>LD
Infantis	25/25	100/>LD	100/>LD	100/>LD
Schwarzengrund	25/25	100/>LD	100/>LD	100/>LD
Worthington	25/50	100/>LD	100/100	100/>LD
Saintpaul	25/25	100/>LD	100/100	100/>LD
Braenderup	25/25	100/>LD	100/>LD	100/>LD
Montevideo	12,5/25	100/>LD	100/>LD	100/>LD
Mbandaka	25/25	100/>LD	100/>LD	50/>LD
Ohio	25/25	100/>LD	100/100	100/>LD
Agona	25/50	100/>LD	100/>LD	50/>LD
Senftenberg	25/50	100/>LD	100/>LD	50/>LD
Corvallis	12,5/12,5	100/>LD	100/100	100/>LD
Hadar	25/25	100/>LD	100/>LD	50/>LD
Grumpensis	12,5/25	100/>LD	100/100	50/>LD
Gafsa	50/50	100/>LD	100/100	50/>LD
Orion	50/50	100/>LD	100/100	50/>LD
Tennessee	12,5/25	100/>LD	100/>LD	50/>LD
Cubana	25/25	100/>LD	100/100	50/>LD
Kentucky	12,5/12,5	100/>LD	100/100	50/>LD
Bareilly	25/50	100/>LD	100/>LD	50/>LD
Livingstone	25/25	100/>LD	100/>LD	50/>LD
Minnesota	12,5/25	100/>LD	100/>LD	50/>LD
Branderburg	25/50	100/>LD	100/100	50/>LD
Enteritidis	25/50	100/>LD	50/100	50/>LD
Newport	25/25	100/>LD	100/100	50/>LD
Entérica	25/25	100/>LD	50/100	100/>LD
Derby	12,5/25	100/>LD	100/100	100/>LD
Heidelberg	25/25	100/>LD	50/100	50/>LD
Typhimurium ATCC	12,5/25	100/>LD	50/100	50/>LD

>LD: Maior que o limite de detecção; Pc: *Plinia cauliflora*; Sm: *Schinus molle*; Pg: *Psidium guajava*; Ma: *Maytenus aquifolium.* *Ocotea spixiana* e *Ricinus communis* não apresentaram atividade antimicrobiana.

Quanto à atividade antimicrobiana d os extratos vegetais da presente pesquisa, um crescente número de mecanismos de ação inibitória tem sido atribuído a compostos ativos

presentes nos produtos naturais. Nas bactérias, a atividade antimicrobiana dos taninos se dá por meio da formação de complexos entre os mesmos e a parede celular, inibindo o transporte de nutrientes e consequentemente retardando o crescimento do micro-organismo (PcSWEENEY et al., 2001). Já os flavonoides e terpenóides possuem atividade decorrente dos efeitos prejudiciais à parede celular bacteriana e consequentemente destruição do micro-organismo (TURINA et al., 2006). A atividade antimicrobiana das cumarinas pode ser atribuída ao fato do anel de cumarina levar à inibição da síntese do ácido nucleico bacteriano (ROSSELLI et al., 2007). Por fim, alguns alcaloides inibem a ação de bactérias gram-negativas causando lise celular e mudanças morfológicas (SAWER et al., 2005), justificando dessa forma a atividade antimicrobiana dos extratos vegetais do presente estudo.

O potencial antimicrobiano de extratos vegetais de *P. cauliflora* já foram reportados por outros pesquisadores (CARVALHO et al., 2009) assim como para *P. guajava* (VARGAS-ALVAREZ et al., 2006), ambas espécies pertencentes a família Myrtaceae, podendo ser atribuída a atividade antimicrobiana dos extratos desses indivíduos devido a presença de taninos, substancia essa amplamente presente na família (LOGUERCIO et al., 2005). O mesmo foi observado que no controle de 20 sorotipos de *Salmonella* os extratos que melhor agiram no controle dos micro-organismos eram espécies da família Myrtaceae (Voss-Rech et al., 2011).

Embora não tenham sido encontrados na literatura trabalhos que explorem a atividade antimicrobiana de *M. aquifolium*, Estevam et al. (2009) reportaram a atividade antimicrobiana do gênero Maytenus frente a bactérias Gram-negativas e Oliveira et al. (2009) afirmaram que o gênero Maytenus possui propriedades fitoquímicas como a presença de terpenoides, taninos, alcaloides e flavonoides, corroborando com os resultados.

Não foram encontradas pesquisas sobre as propriedades farmacológicas dos extratos aquosos ou etanólicos de *S. molle*. Porém, Carvalho et al. (2013) relataram que os compostos marjoritários na família Anarcadiaceae são os terpenoides e flavonoides. Além destes compostos, a triagem fitoquímica da presente pesquisa constatou a presença de taninos (Tabela 1).

A atividade inibitória de *R. communis* pode estar relacionada aos seus constituintes como ricina e flavonoides (HENRIQUES et al., 2002) além da presença de taninos e triterpenoides (Tabela 1). Contudo, um dos problemas associados à utilização do extrato de mamona refere-se a presença da ricina, uma vez que esta é venenosa a humanos e insetos (LER, et al, 2006), sugerindo-se então o isolamento de compostos ou detoxicação do extrato antes do seu uso.

Não foram encontrados na literatura estudos fitoquímicos de extratos de *O. spixiana*, entretanto, Zanin & Lordello (2007) reportaram a presença de compostos alcaloides em

cinquenta e quatro espécies do gênero Ocotea, podendo estar relacionado a atividade antimicrobiana apresentada por esta espécie.

Dentre os extratos avaliados, o maior efeito bacteriostático foi obtido para o extrato etanólico de *P. cauliflora* seguido por *P. guajava*. Comparando o perfil fitoquímico dessas espécies com as demais, observa-se que foram as únicas que possuem em sua composição cumarinas. A elevada atividade antimicrobiana das cumarinas é devido a sua característica lipofílica e estrutura molecular planar, que contribuem na penetração da mesma na membrana celular bacteriana ou parede celular (Kayser e Kolodzie, 1999).

De maneira geral, os extratos etanólicos apresentaram melhor atividade inibitória quando comparados aos extratos aquosos. Devido à diferença de polaridade, a extração aquosa e etanólica podem conferir a extração de quantidades diferentes dos metabólitos, sendo a água capaz de extrair em sua maioria compostos como antocianinas, amidos, taninos, saponinas, terpenoides, polipeptídeos e lecitinas, já o álcool, por sua vez, é responsável pela extração além de taninos e terpenóides também de polifenois, poliacetilenos, esteróis, alcaloides e os flavonoides (COWAN, 1999). Isto explica o fato de que em uma mesma planta, diferentes extratos apresentarem resultados distintos. Além disso, a composição dos extratos pode também variar de acordo com as condições ambientais, estações do ano em que foram coletadas, bem como, às diferentes técnicas empregadas para avaliação da atividade, por não haver uma padronização internacional para avaliação de extratos vegetais (ALVES et al., 2008). Desta forma, é notória a necessidade da padronização de técnicas para avaliar a atividade de extratos vegetais com o intuito de corroborar e assegurar os resultados encontrados.

A suscetibilidade microbiana aos extratos vegetais testados no presente estudo variou dependendo do sorotipo, conforme descrito anteriormente por Voss-Rech et al., (2004), que testaram a suscetibilidade de *Salmonella* spp. frente a diferentes extratos vegetais. A explicação pode ser devido às pressões seletivas que os sorotipos podem sofrer de acordo com a utilização de diferentes antimicrobianos, desenvolvendo resistências (WHO, 2000).

As propriedades antimicrobianas e antioxidantes de extratos vegetais tem despertado interesse pela perspectiva de constituírem uma alternativa para as exigências dos consumidores quanto à utilização de aditivos naturais em diferentes produtos (TASSOU et al., 2000), destacando-se nesse sentido os frangos, por ser um dos alimentos base da alimentação. Sendo assim, os resultados obtidos comprovam que em sua maioria os extratos etanolicos e aquosos das diferentes espécies brasileiras testadas apresentam atividade antimicrobiana e antioxidante, demonstrando assim o potencial uso no desenvolvimento de produtos de origem natural. O uso de folhas permite a obtenção de matéria-prima sem o corte

da planta fazendo com que o cultivo e uso da espécie seja viável, sem a necessidade de explorar populações nativas que se encontram ameaçadas.

CONCLUSÃO

Os extratos etanólicos e aquosos das folhas das espécies avaliadas apresentaram em sua maioria as mesmas classes de metabólitos secundários para todas as plantas, sendo eles, taninos, flavonoides e triterpenoides, com exceção dos extratos de *P. cauliflora* e *P. guajava* que apresentaram as cumarinas e *O. spixiana* e *M. aquifolia* que apresentam alcalóides além dos metabólitos citados anteriormente. Em relação a atividade antioxidante os extratos etanólico de *M. aquifolia*, *P. guajava* e aquoso de *O. spixiana* foram os melhores, seguido do extrato aquoso de *M. aquifolia* e etanólico de *P. cauliflora.* Os extratos testados apresentaram atividade inibitória em diferentes concentrações sobre os sorotipos de *Salmonella* variando de acordo com o solvente extrator e o sorotipo testado, sendo o extrato etanólico de *P. cauliflora* seguido por *P. guajava* de maior efeito bacteriostático.

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ANEXOS

Capítulo 1: Chemical Composition and Antimicrobial and Antioxidant Activity of Essential Oil and Various Plant Extracts from *Prunus myrtifolia* (L.) Urb

- Organizado de acordo com a Revista “African Journal of Agricultural Research”.

Capítulo 2: Composição química, atividade antioxidante e antimicrobiana de extratos vegetais de seis espécies vegetais frente a sorotipos de *Salmonella* de origem aviária.

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