ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Antibacterial activity of avocado extracts (*Persea americana* Mill.) against *Streptococcus agalactiae*

Actividad antibacteriana de extractos de aguacate (*Persea americana* Mill.) sobre *Streptococcus agalactiae*

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Abstract. Plants contain numerous constituents and are valuable sources of new biologically active molecules. Avocado (*Persea americana* Mill.) is cultivated and used as food in most tropical and subtropical countries. Its high nutritional value and biological activities, as antioxidant, antimicrobial and analgesic properties, have been thoroughly investigated. Interest in plant extracts with antimicrobial properties has increased as a result of the indiscriminate use of antibiotics, leading to the emergence of resistant bacterial strains. Among bacterial species with clinical importance to multiple hosts, *Streptococcus agalactiae* is outstanding, as it can cause infections especially in humans, fish and cattle. The current study aimed to evaluate the antimicrobial activity of two extracts (ethanol and dichloromethane) from avocado seeds, 'Margarida' variety, against isolates of *S. agalactiae*. Extracts were diluted in ethanol / water (1:1) at a concentration of 100 mg/mL. Antimicrobial activity was tested by the disk diffusion method (antibiogram) against isolates of *S. agalactiae* of human and fish origin.The ethanol extract showed antimicrobial activity only for some isolates of *S. agalactiae* of human origin. The dichloromethane extract showed activity against all isolates of S. agalactiae of both origins. A comparison of the results obtained with dichloromethane extract from isolates of *S. agalactiae* of human or fish origin demonstrated the existence of phenotypic variability among isolates from the same host. However, when comparing measurements obtained in each of the groups, they were statistically similar, showing a lack of interpopulation variability. Thus, it can be verified that the resistance profile of isolates of *S. agalactiae* was independent of host origin and typical of the species.

Keywords: Plant extracts; Disk diffusion method.

Resumen. Las plantas contienen numerosos constituyentes y son fuentes ricas de nuevas moléculas biológicamente activas. El aguacate (*Persea americana* Mill.) es cultivado y utilizado como alimento en la mayoría de los países tropicales y subtropicales, ya que tiene alto valor nutricional, y sus actividades biológicas han sido muy investigadas, entre las cuales la actividad antioxidante, analgésica o antimicrobiana. El interés en extractos vegetales con propiedades antimicrobianas se ha intensificado como consecuencia de la utilización indiscriminada de antibióticos, que llevó a la selección de cepas bacterianas resistentes. Entre las especies bacterianas de relevancia clínica para variados hospederos, se puede destacar la bacteria *Streptococcus agalactiae*, que puede causar infecciones, principalmente en humanos, peces y ganado. El objetivo de ese trabajo fue evaluar la actividad antimicrobiana de dos extractos (etanólico y diclorometanico) de hueso de aguacate variedad margarita en relación a aislados de *S. agalactiae*. Los extractos fueron resuspendidos en etanol/agua en la concentración de 100 mg/mL. La actividad antibacteriana de los extractos fue comprobada usando el método de difusión en discos frente a aislados de *S. agalactiae* de origen humano y peces. El extracto etanólico presentó actividad antimicrobiana solamente para algunos aislados de *S. agalactiae* de origen humano. El extracto diclorometanico presentó actividad antimicrobiana para todos los aislados de *S. agalactiae* de ambos orígenes. La comparación de los resultados obtenidos con el extracto diclorometanico enfrente a los aislados de *S. agalactiae* de origen humano y peces mostró la existencia de variabilidad fenotípica entre aislados del mismo hospedero. Sin embargo, la comparación de las medias obtenidas en cada uno de los grupos fue estadísticamente semejante, demostrando la ausencia de variabilidad interpoblacional. De esta manera, se pudo observar que el perfil de resistencia de aislados de *S. agalactiae* fue independiente del hospedero de origen y característico de la especie.

Palabras clave: Extractos vegetales; Método de difusión en disco.

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Received 17.X.2014. Accepted 17.IV.2015.

INTRODUCTION

The control of bacterial infections is mostly carried out with antibiotics. However, the emergence of resistant bacterial strains has become more frequent, leading to the need of new sources of molecules with antimicrobial activity, which have been found mainly in microorganisms and plants (Cowan, 1999; Mlynarczyk et al., 2010). Natural plant products have been used since ancient times for medicinal purposes as they comprise numerous components and valuable sources of new biologically active molecules (Cowan, 1999; Gupta et al., 2004).

Many plants synthesize antimicrobial secondary metabolites as part of their normal growth and development, often keeping them in tissues that need protection against microbial attack (Gupta et al., 2004). The antimicrobial activity of plant extracts may reside in a variety of different phytochemical constituents, namely terpenoids, essential oils, alkaloids, lectins, polypeptides and polyphenolics and phenolic substances (simple phenols, phenolic acids, quinones, flavones, flavonols and flavonoids, tannins and coumarins) (Gonçalves et al., 2005). The antibacterial activity of these extracts may be ascribable to the combined effects of the polyphenols adsorption on bacterial membrane, leading to its rupture and subsequent leakage of cellular content, and the generation of hydroperoxides (Negi, 2012).

Among plants, avocado (*Persea americana* Mill), originated from Central America, presents a high nutritional value and is cultivated and used as food in most tropical and subtropical countries. Its peel, fruit and leaves are commonly used in America, Antilles and Africa for the treatment of various diseases such as menorrhagia, hypertension, stomach pain, bronchitis, diarrhea and diabetes (Adeyemi et al., 2002). However, avocado seeds are usually discarded during consumption or industrial processes generating residues that could be an economical alternative for treatment of some diseases.

The avocado leaf, stem, fruit and peel have biological activities scientifically proven (Miranda et al., 1997; Adeyemi et al., 2002; Quing-Yi et al., 2005; Gomez-Flores et al., 2008; Castro et al., 2010; Rodríguez-Carpena et al., 2011). Studies with seed demonstrated antioxidant activity and antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas* spp. and *Yarrowia lipolytica*. The Gram-positive bacteria are more sensitive than Gram-negative bacteria (Rodríguez-Carpena et al., 2011). Other seed properties already studied are larvicidal (in *Aedesaegypti*), antifungal (*Candida* spp., *Cryptococcus neoformans* and *Malassezia pachydermatis*) (Leite et al., 2009) and antimicrobial activities against several species including *S. aureus, Enterococcus faecalis, Salmonella Enteritidis, Citrobacter freundii, Pseudomonas aeruginosa, Salmonella Typhimurium, Enterobacter aerogenes* and *Zygosaccharomyces bailii* (Chia & Dykes, 2010).

Phytochemical studies of the avocado seed allowed the identification of several classes of active compounds such as flavonoids, anthocyanins, condensed tannins, alkaloids and triterpenoids in methanolic extracts, while sterols and triterpenes were detected in the hexane extract (Leite et al., 2009).

Several bacterial species are considered of clinical importance because they cause a number of diseases in various hosts. Among these, the species *Streptococcus agalactiae,* a Gram positive, catalase negative and facultatively anaerobic bacteria is remarkable. This species can cause infections in cattle, humans and fish. Furthermore, it can occasionally infect mice, cats, dogs, camels and frogs (Elliot et al., 1990; Figueiredo, et al., 2006; Pereira et al., 2010).

Streptococcus agalactiae is one of the most common causes of perinatal bacterial infections in humans. It is also an opportunistic pathogen of the elderly and immunocompromised people, and may cause pneumonia, meningitis, bacteremia and skin or soft tissue infections (Gibbs et al., 2004; Nakamura et al., 2011). Penicillin is the treatment of choice. However, for patients allergic to β-lactam, erythromycin or clindamycin are prescribed. Mammal isolates are preferably β-hemolytic, but some nonhemolytic have been isolated, and are usually cultivated at 37 °C (Evans et al., 2002; Gibbs et al., 2004).

Besides humans, *S. agalactiae* can infect freshwater and marine fish, either in fish farming or free in the environment (Figueiredo et al., 2006). It is considered the main pathogenic bacteria of different species of fish with high mortality. Naturally or experimentally infected fish exhibit symptoms, such as unilateral or bilateral exophthalmia, corneal opacity, erratic swimming, changes in skin color, skin lesions and ascites (Figueiredo et al., 2006; Pretto-Giordano et al., 2010a). Fish isolates of *S. agalactiae* are usually not hemolytic and are cultivated at 30°C, which may indicate phenotypic adaptations to host (Elliot et al., 1990; Evans et al., 2002; Castro et al., 2008).

S. agalactiae isolates of human source present resistance to tetracycline, clindamycin, erythromycin, chloramphenicol, rifampicin, norfloxacin, levofloxacin, ciprofloxacin, moxifloxacin (Borger et al., 2005; Correa et al., 2011; Nakamura et al., 2011; Ki et al, 2012; Usein et al., 2012). Fish isolates may be resistant to nalidixic acid, gentamicin, neomycin, norfloxacin and streptomycin (Evans et al., 2002; Figueiredo et al., 2006).

The susceptibility of *S. agalactiae* to natural extracts was analyzed in different works. According to Cueva et al. (2012), *S. agalactiae* presented sensitivity to phenolic compounds isolated from wine, epicatechin and gallic acid, and was not sensitive to oenological extracts. It was also sensitive to extracts of wild mushrooms (Alves et al., 2012) and to the essential oil from eight eucalyptus species (Elassi et al., 2012). Leaf extracts of *Calyptranthes clusiifolia*, *Croton floribundus*, *Heisteria silvianii*, *Merremia tomentosa* and *Zanthoxylum riedelianum* also inhibited *S. agalactiae* growth (Castro et al., 2008).

Thus, the aim of this study was to investigate the antibacterial activity of avocado (*P. americana* Mill) seed extracts against

S. agalactiae isolates of human and fish origin. Therefore, comparison of intra- and inter-population variability of resistance profiles was evaluated and indicated the potential therapeutic use of the avocado seed against this bacterial species.

MATERIALS AND METHODS

Plant extracts. In order to obtain seed extracts of avocado (*P. americana* Mill., 'Margarida' variety), the seed was initially separated from the pulp, fragmented, dried and ground into powder. The seed powder was then exposed to a maceration process for a period of seven days, either using ethyl alcohol as solvent, resulting in an extract termed "ethanolic extract", or using dichloromethane as solvent, yielding an extract termed "dichloromethane extract". Subsequently, the extracts were filtered and concentrated in a rotary evaporator. Procedures of maceration, filtration and concentration were repeated once more with both extracts. In order to measure the efficiency of extraction, the obtained extracts were weighed and the ratio between 500g of the initial seed powder and the final weight calculated. Extracts were dissolved in ethanol / water (1:1), stored at room temperature and protected from light until use.

Bacterial strains and culture conditions. The evaluation of 29 *S. agalactiae* isolates recovered from vaginal-rectal swabs and urine of female patients at the University Hospital of Universidade Estadual de Londrina (originally used by Otaguiri et al., 2013) was performed. These isolates had already been characterized for bacterial species confirmation by phenotypic tests (CAMP, KEA, NaCl, hippurate, bacitracin, trimethoprim-sulfamethoxazole, Gram staining and catalase). These isolates were incubated for 24 hours at 37 °C in Muller Hinton blood agar plates (supplemented with 5% sheep blood).

The assessment of 26 isolates of *S. agalactiae* obtained from the Nile tilapia (*Oreochromis niloticus*) with bacterial infection symptoms was conducted. The isolates were collected from different organs, including eyes, brain, liver, heart, blood, visceral fluid and kidney fish collected at fish farming properties located in the northern region of Paraná state and northwest region of São Paulo state, Brazil.The strains had been previously identified as *S. agalactiae* by Gram stain and biochemical assays, and confirmed by more accurate tests, such as API 20 Strep Microtest (BioMerieux) and SlidexStrepto-kit (BioMerieux) (Pretto-Giordano et al., 2010b). These isolates were incubated for 48 hours at 30 °C in Muller Hinton blood agar plates.

Antibiograms. The antibacterial activity of avocado seed extracts against *S. agalactiae* was evaluated by the Disc diffusion method on Muller Hinton blood agar plates, as recommended by CLSI (Clinical Laboratory Standard Institute, 2010). For this purpose, bacteria concentration followed the 0.5 MacFarland scale, yielding an inoculum density of approximately 10^8 CFU/mL (Ostrosky et al., 2008) which was homogeneously distributed over the plates using sterile swabs.

Discs of 6 mm diameter (Laborclin, Brazil) received the application of 10 µL of 100 mg/mL ethanol or dichloromethane extracts. Additionally, other discs received 10 µL of solvents and were used as a negative control. All discs were kept for an hour under a laminar flow for solvent evaporation (Ostrosky et al., 2008).

Biplates were used, forming a duplicate of each isolate per plate. Three discs were placed on each plate side: control, ethanol and dichloromethane extract. In other words, two disks were tested for each extract per strain. Samples of human source were incubated at 37 °C for 24 hours. Strains of fish origin were maintained at 30 °C for 48 hours. At the end of this time, the inhibition zone diameter was measured.

Statistical analysis. The susceptibility test results were analyzed using the Analysis of Variance (ANOVA) followed by the Tukey test or the Mann-Whitney test for interpopulation analysis, at 95% confidence level. Tests were performed with the GraphPad InStat program, version 3.05.

RESULTS

After the extraction procedures, the final weight of extracts was 12.76 g for the ethanolic and 7.48g for the dichloromethane extract. Bacterial inhibition by extracts was evaluated visually by measuring the inhibition zone diameters around disks (disk diameter included) recorded in millimeters. The antimicrobial activity was classified into three levels: low activity (inhibition zone ≤12 mm), moderate activity (inhibition zone between 12 and 20 mm) and strong activity (inhibition zone ≥20 mm), following the criteria adopted in other studies with plant extracts (Rota et al., 2008; Fei et al, 2011).

Antibiogram results of *S. agalactiae* isolates are shown in Table 1 and exemplified in Figures 1 and 2. Both human and fish isolates showed statistical variability in intra-group analysis, exhibiting an inhibition zone between 7 mm e 13 mm for human isolates, and between 9 mm and 12 mm for fish isolates.

For the intergroup analysis, the average of inhibition zones obtained for each group (human and fish origin) was compared. Statistical analysis for ethanolic extract could not be performed, since inhibition zones on plates with fish isolates were not observed. However, differences in susceptibility between strains of human and fish could be observed, given that the first show some susceptible isolates, while in the latter, no susceptible isolates were found (Table 1). The antimicrobial activity of the ethanolic extract, when present, was considered weak, with an inhibition zone between 7 mm and 9.5 mm.

The mean ± standard deviation of the *inhibition zone* diameter for the isolates of human origin observed for the dichlo-

Table 1. Antimicrobial activity of avocado seed extracts against *S. agalactiae* strains.
Tabla 1. Actividad antimicrobiana de extractos de semilla de aguacate contra cepas de *S. agalactiae*.

Isolate (human source)	Inhibition zone diameter (mm)		Isolate (fish source)	Inhibition zone diameter (mm)	
	Ethanolic extract	Dichloromethane extract		Ethanolic extract	Dichloromethane extract
	Mean ± Standard Deviation	Mean ± Standard Deviation		Mean ± Standard Deviation	Mean ± Standard Deviation
6	7.75 ± 0.35	10.75 ± 0.35	**15**	0.00 ± 0.00	10.75 ± 0.35
9	7.75 ± 0.35	11.75 ± 0.35	**16**	0.00 ± 0.00	10.50 ± 0.00
10	4.00 ± 5.66	11.00 ± 0.00	**18**	0.00 ± 0.00	10.75 ± 0.35
11	8.75 ± 1.06	11.00 ± 0.71	**19**	0.00 ± 0.00	11.00 ± 0.00c
12	0.00 ± 0.00	11.00 ± 0.00	**23**	0.00 ± 0.00	11.00 ± 0.00c
13	0.00 ± 0.00	11.25 ± 0.35	**25**	0.00 ± 0.00	10.75 ± 0.35
14	0.00 ± 0.00	12.75 ± 0.35	**26**	0.00 ± 0.00	11.25 ± 0.35 c.e
21	0.00 ± 0.00	11.25 ± 1.06	**29**	0.00 ± 0.00	10.50 ± 0.00
24	0.00 ± 0.00	10.75 ± 0.35	**30**	0.00 ± 0.00	10.75 ± 0.35
25	0.00 ± 0.00	11.25 ± 0.35	**34**	0.00 ± 0.00	10.50 ± 0.00
26	0.00 ± 0.00	10.25 ± 0.35 a	**35**	0.00 ± 0.00	9.75 ± 0.35 d
27	0.00 ± 0.00	10.50 ± 0.00 a	**37**	0.00 ± 0.00	9.75 ± 0.35 d
28	0.00 ± 0.00	10.25 ± 0.35 a	**38**	0.00 ± 0.00	11.00 ± 0.00 c
29	0.00 ± 0.00	10.50 ± 0.00 a	**39**	0.00 ± 0.00	10.25 ± 0.35
33	4.25 ± 6.01	10.50 ± 0.71 a	**40**	0.00 ± 0.00	9.25 ± 0.35d
37	7.25 ± 0.35	11.00 ± 0.00	**42**	0.00 ± 0.00	11.75 ± 0.35 c.e
42	3.50 ± 4.95	11.25 ± 0.35	**44**	0.00 ± 0.00	10.75 ± 0.35
43	0.00 ± 0.00	11.75 ± 1.06	**45**	0.00 ± 0.00	11.25 ± 0.35 c.e
49	0.00 ± 0.00	10.50 ± 0.00 a	**46**	0.00 ± 0.00	10.75 ± 0.35
50	0.00 ± 0.00	11.00 ± 0.71	**47**	0.00 ± 0.00	10.50 ± 0.00
52	0.00 ± 0.00	12.00 ± 0.00	**48**	0.00 ± 0.00	9.50 ± 0.00 d
54	0.00 ± 0.00	10.25 ± 0.35 a	**50**	0.00 ± 0.00	10.50 ± 0.71
56	0.00 ± 0.00	9.75 ± 0.35 a.b	**52**	0.00 ± 0.00	11.00 ± 0.71 c
59	0.00 ± 0.00	11.00 ± 0.71	**53**	0.00 ± 0.00	10.25 ± 0.35
60	0.00 ± 0.00	10.25 ± 0.35 a	**55**	0.00 ± 0.00	10.75 ± 1.06
61	3.50 ± 4.95	10.25 ± 0.35	**56**	0.00 ± 0.00	11.00 ± 0.00 c
62	0.00 ± 0.00	11.00 ± 0.00			
70	0.00 ± 0.00	11.00 ± 0.00			
96	0.00 ± 0.00	11.25 ± 1.06			

a: statistically differs from strain 14; b: statistically differs from strain 52; c: statistically differs from strain 40; d: statistically differs from strain 42; e: statistically differs from strain 48 (P<0.05).
a: difiere estadísticamente de la cepa 14; b: difiere estadísticamente de la cepa 52; c: difiere estadísticamente de la cepa 40; d: difiere estadísticamente de la cepa 42; e: difiere estadísticamente de la cepa 48 (P<0,05).

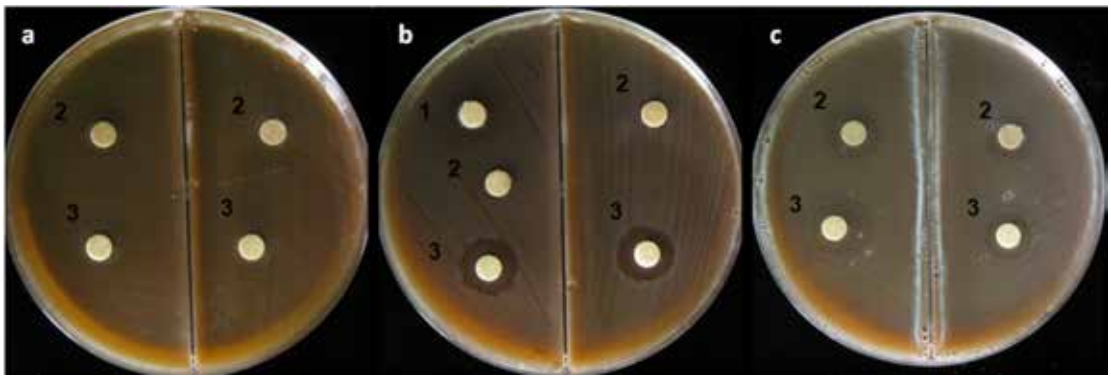

Fig. 1. Antibiogram of *S. agalactiae* isolates of human source: a) strain 6, b) strain 14 and c) strain 25. Disc 1: ethanol/water control; Disc 2: ethanolic extract (100 mg/mL); Disc 3: dichloromethane extract (100 mg/mL).
Fig. 1. Antibiograma de aislados humanos de *S. agalactiae*: a) cepa 6, b) cepa 14 y c) cepa 25. Disco 1: control etanol/agua; Disco 2: extracto etanólico (100 mg/mL); Disco 3: extracto diclorometanico (100 mg/mL).

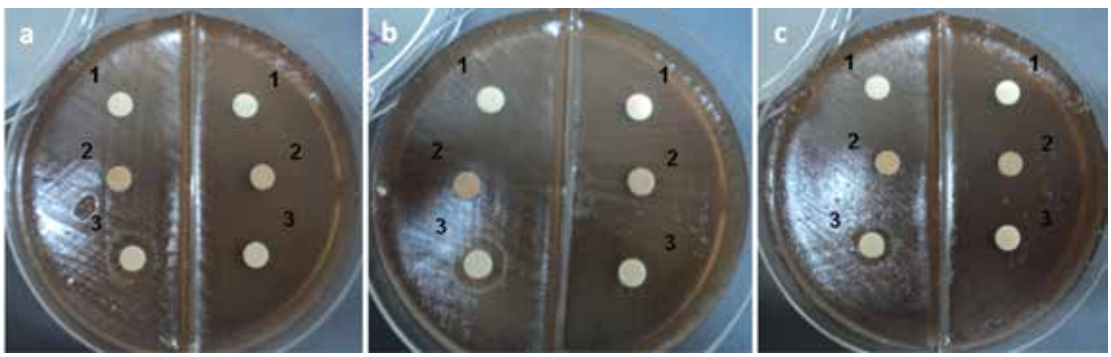

Fig. 2. Antibiogram of *S. agalactiae* isolates of fish source: (a) strain 26, (b) strain 34 and (c) strain 55. Disc 1: ethanol/water control; Disc 2: ethanolic extract (100 mg/mL); Disc 3: dichloromethane extract (100 mg/mL).
Fig. 2. Antibiograma de aislados de peces de *S. agalactiae*: (a) cepa 26, (b) cepa 34 y (c) cepa 55. Disco 1: control etanol/agua; Disco 2: extracto etanólico (100 mg/mL); Disco 3: extracto diclorometanico (100 mg/mL).

romethane extract was 10.93 ± 0.62 mm. On the other hand, fish isolates presented a mean ± standard deviation of 10.61 ± 0.56 mm. The comparison between means was not statistically significant, with p = 0.0897. The dichloromethane extract antibacterial activity was considered weak.

DISCUSSION

Plant extracts are sources of a variety of biotechnology products. Therefore, countless studies have been conducted in order to evaluate characteristics of these extracts, which can be used for the treatment of diseases, due to their antimicrobial, antifungal, analgesic, anti-inflammatory and antitumor activities (Miranda et al., 1997; Adeyemi et al., 2002; Qing-Yi et al., 2005; Leite et al., 2009; Rodríguez-Carpena et al., 2011). Among the commonly evaluated properties, the antimicrobial activity has received special attention, and numerous studies have been conducted, including different avocado extracts (Gomez-Flores et al., 2008; Castro et al., 2010; Chia & Dykes, 2010; Rodríguez-Carpena et al., 2011).

However, although widely used, there are not yet any standardization methods to analyze the antimicrobial activity of extracts of natural products (Ostrosky et al., 2008). The Disk diffusion test is indicated by the FDA (Food and Drug Administration / USA) and established as standard by the

CLSI (Clinical Laboratory Standard Institute / USA, 2010), and, therefore, was the method chosen to conduct this study.

Several *S. agalactiae* isolates of human and fish origin were used in this work, aiming to comprise different phenotypic variations found in isolates from each of the two sources, as well as verify what kind of host presents isolates more susceptible to the evaluated extracts.

Human source isolates used in this study have already been analyzed for capsular type, genotyping by MLVA, antibiotics susceptibility and genetic virulence determinants. The results suggest that even commensal *S. agalactiae* isolates have high potential for virulence and are susceptible to most antimicrobial agents tested (penicillin, ampicillin, vancomycin, etc.) (Otaguiri et al., 2013). However, they presented moderate resistance to erythromycin (19%) and clindamycin (13%) which demands the search for new treatment alternatives, especially for patients allergic to β-lactam antibiotics.

The difference in efficiency of the two extracts can be explained by the difference in polarity of solvents. During the extraction process, polarity influences solubility of the main active substance, leading to difference in their chemical composition and consequently, in their biological activity (Idris et al., 2009). The yield of extraction and concentration of the extract solution can also intervene in the results.

The antimicrobial activity of avocado extracts may be ascribable to its chemical composition. Phytochemical screening highlighted the presence of phenolic compounds in avocado tissues, whose antimicrobial activity is well documented (Idris et al., 2009; Rodriguez-Carpena et al., 2011).

Avocado seed extracts showed low antimicrobial activity against of *S. agalactiae* isolates. This can probably be overcome by increasing extract concentration. The results indicate that the avocado seed is a potential source of antimicrobial substances and arouses considerable interest in new research with more purified extracts for the identification of compounds responsible for the antimicrobial activity.

ACKNOWLEDGEMENTS

This work was supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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