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Antioxidant and antimicrobial activity of *Kielmeyera coriacea* Mart. and Zucc

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Kielmeyera coriaceae **Mart. and Zucc. belongs to Clusiaceae family and is largely distributed in the cerrado Brazilian biome. In this study, phytochemical screening, the antioxidant and antimicrobial activities of aerial parts of the *K. coriacea* were evaluated. The extracts showed the presence of terpenoids, saponins, triterpenes, steroids, flavonoids, tannins and phenolic compounds. Ethanolic extract and partitions of plant parts of *K. coriacea* showed a very strong antioxidant activity, in particular, the inner bark of the plant. The ethanolic extract and the cyclohexane fraction of the inner bark showed high antimicrobial activity against oral pathogens with minimum inhibitory concentrations (MIC) between 3.1 and 100 µg ml^{-1}. The cyclohexane fraction was the most active with CIMs of 6.2 µg ml^{-1} for most of the tested bacteria, including *Streptococcus mutans*, the main etiological agent of dental caries. The ethanolic extract exhibited the highest antibacterial activity against *Streptococcus mitis*, with MIC value of 3.1 µg ml^{-1}, a value lower than that found by the positive control. The antioxidant and antimicrobial activities could be due to the different phytochemical classes presented in the ethanolic extract and partitions studied. The antioxidant activity and the antimicrobial activity showed by the ethanolic extract and cyclohexene fraction of the inner bark were considered promising.**

Key words: *Kielmeyera coriacea*, Clusiaceae, extracts phytochemical, antioxidant, antimicrobial activities.

INTRODUCTION

The Cerrado is the second largest biome found in Brazil, providing about 160,000 species of plants, animals and fungi (Brannstrom et al., 2008) and is recognized as the richest savanna in the world, housing 11,627 native plant species already cataloged (MMA, 2013), having 220 species with medicinal use. Among these species is the *Kielmeyera coriacea* Mart. and Zucc, largely distributed in the cerrado and whose aqueous extract is used to treat various tropical diseases such as malaria, schistosomiasis, leishmaniasis, bacterial and fungal infection (Ferri, 1969; Zagoto et al., 2006; Audi et al., 2002). Furthermore, the resin extracted from the bark of *K. coriacea* is used in folk medicine for treatment of dental pain (Pinto et al, 1993; Corrêa, 1952). The genus

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Kielmeyera belongs to the family Clusiaceae, the second richest genera in Brazil, with 46 species and two subspecies with large distribution (Bittrich, 2010). Dichloromethane extracts of *K. coriacea* leaves and stems are rich in xanthones, triterpenes and biphenileted compounds (Cortez et al., 2002; Cortez et al., 1998). Some compounds isolated from the dichloromethane extract of leaves exhibited antimicrobial activity (Cortez et al., 2002), antifungal activity (Cortez et al. 1998) and the mixture of δ-tocotrienol, and dimers found in hexane extract of the root bark from *K. coriacea* presented, *in vitro*, action against strains of cancer (Mesquita et al., 2011). The effect on the central nervous system of the hydroalcoholic extract of leaves and steam of *K. coriacea* was evaluated exhibiting a substantial anxiolytic effect (Audi et al., 2002; Otobone et al., 2007). Studies on antimicrobial activity of extracts of other *Kielmeyera* species are known. The ethyl acetate and ethanolic extracts from *Kielmeyera neglecta* have shown activities against multidrug-resistant bacteria *Enterococcus faecalis* and *Staphylococcus aureus* (Adrioli et al., 2012). The dichloromethane extract of the stem of *Kielmeyera cuspidata* exhibited antibacterial activity for the microorganisms *Micrococcus luteus*, *Bacillus subitilis*, *Staphylococcusaureus* and *Streptococcus mutans* (Sobral et al., 2009).

To the best of our knowledge, there is no previous report on the antioxidant and antimicrobial activity against oral pathogens of extracts and partitions of the specie *K. coriacea*. So, the main objective of this work was evaluating these biological activities of the aerial parts of the *K. coriacea*.

MATERIALS AND METHODS

Plant materials

Aerial parts of the *K. coriacea* Mart. & Zucc**.** were collected in Jardim Karaíba district, located in the city of Uberlândia, Minas Gerais state, Brazil (18°94'08.27"S, 48°26'06.40"W), in April 2010. The plant material was identified properly by a specialist (Prof. Glein Monteiro de Araújo, Institute of Biology-UFU), and a voucher specimen (57181) of the plant was deposited in the herbarium of the Federal University of Uberlândia, Brazil.

Preparation of extracts

Aerial parts of *K. coriacea* were air-dried, cleaned, cut into small pieces, peeled**,** minced, and subsequently milled. 100 g of each powdered material (leaves, wood, inner bark, and outer bark) was weighed, homogenized and extracted with 500 ml of ethanol (95% purity) as solvent using Soxhlet apparatus, and subjected to continuous hot percolation for 6 h (Hargeman et al. 1998). Each crude extract was decanted, filtered under vacuum, and then concentrated by a rotary evaporator at 40°C, to give a viscous dark mass. Each dried sample was weighed, yielding (%) 20.47±0.6 of leaves, 16.38±0.1 of inner bark, 7.47±0.3 of outer bark and 0.54±0.2 of wood extract. They were transferred to amber flasks and stored at -18 ± 5°C for further analysis.

Liquid-liquid partition of ethanol extracts

A sample (10 g) of the crude ethanolic extract of each aerial plant parts was resuspended in 200 ml of methanol/water (9:1). Liquid-liquid partition was performed using a sequence of solvents (cyclohexane, dichloromethane, ethyl acetate and methanol). In total, 600 ml of each of the solvents (3 x 200 ml) were used. The solutions of cyclohexane, dichloromethane, ethyl acetate and methanol with their extraction had their solvent evaporated under reduced pressure, at a temperature of 40°C.

Phytochemical screening

The preliminary qualitative phytochemical tests of different extracts of *K. coriacea* were analyzed and the methods were described by Wagner and Bladt (1996). 1000 ppm solution in methanol of each sample was used for the screening. The following phytoconstituents, namely, alkaloids, amino acids, terpenoids, saponins, triterpenes, steroids, flavonoids, tannins and phenolic compounds were screened. The samples were applied to the TLC plates and a positive result was given by the following tests:

1) Detection of alkaloids: the alkaloids appear as brown or orange-brown zones immediately spraying Dragendorff reagent.
2) Detection of amino acids: the amino acids appear as a red-violet color with ninhydrine.
3) Detection of terpenoids: immediately after spraying phosphomolybdic acid the constituents show uniform blue zones.
4) Detection of saponins: inspection under UV-365 nm light results in blue, violet and green fluorescent zones with Anisaldehyde-sulphuric acid reagent.
5) Detection of triterpenes and steroids: development of fluorescence under UV-365 nm light indicates the presence of sterols and triterpenes with Liebermann-Burchard reagent.
6) Detection of flavonoids: typical intense fluorescence in UV-365 nm as orange or yellow colors is produced immediately on spraying NP/PEG reagent.
7) Detection of tannins and phenolic compounds: the compounds can be identified by ferric chloride reagent with the formation of color patches Prussian blue.

Antioxidant activity

Determination of total phenolic content

The total phenolic content was determined using the modified Folin-Ciocalteau reagent method (Singleton et. al., 1965; Sousa et. al 2007) and gallic acid as standard. 12.5 mg of the test sample were transferred quantitatively into a volumetric flask and the final volume (50.0 ml) was completed with methanol. An aliquot of 0.5 ml was transferred to a test tube. Then, 2.5 ml of a solution of Folin-Ciocalteu reactive (10% **v** $\mathbf{v^{-1}}$) and 2.0 ml of (7.5% **v** $\mathbf{v^{-1}}$**)** Na_2CO_3 solution were added. This mixture was kept in a water bath at a temperature of 50°C for 5 min. The absorbance was recorded at 760 nm in a UV-Vis U-300 (Hitachi, Kyoto, Japan) spectrophotometer. Gallic acid was used for the construction of a standard curve at various concentrations (10, 20, 30, 40, 50, 60, 70 and 80 µg ml^{-1}). The total phenolic content was determined by interpoling the samples absorbance values against a calibration curve constructed from standards of gallic acid (100 to 1000 µg ml^{-1}). The results were expressed as mg of gallic acid equivalents (GAE) g^{-1} of dry extract (Table 2). The analyses were performed in triplicate.

Determination of total proanthocyanidins

The total proanthocyanidin content was determined by the vanillin method with modifications (Godefroot et al., 1981). An aliquot of 2 ml from the solution prepared for the total phenolic content was transferred to a test tube. Then, 3 ml of a freshly prepared solution of vanillin in sulphuric acid (70% v v^{-1}) solution was added. The resulting solution (5000 µg ml^{-1}) was kept in a water bath at a temperature of 50°C, for 15 min. The sample was cooled and the absorbance was recorded at 500 nm in a UV-Vis U-300 (Hitachi, Kyoto, Japan) spectrophotometer. The content of tannins was determined by interpolating the samples absorbance values against a standard curve constructed from standards of catechin (100 to 1000 µg ml^{-1}). The standard curve was constructed under the same reaction conditions where the sample was replaced by catechin. The results were expressed as mg of catechin equivalents (CE) g^{-1} of dry extract (Table 2). The analyses were performed in triplicate.

***Quantitative analysis of antioxidant activity* (EC_{50})**

The antioxidant activity was determined following the method described by Brand-Williams et al (1995). Stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, ≥ 85%; FLUCKA) was used for the construction of a standard curve (dilutions of 50, 45, 40, 35, 30, 25, 20, 15, 10, 5 and 1 µg ml^{-1} were used). The absorbance measurements were performed in triplicate with 1 min of intervals between each reading. For the preparation of aqueous solutions, ultra-purified water (Millipore Milli-Q system with resistivity ≥ 18 MΩ cm) was used. The ethanolic extracts or partitions solutions (500 µg ml^{-1}) of each plant part of *K. coriacea* and the positive control were diluted in methanol at concentrations of 250 to 25 µg ml^{-1} and then added to 3.9 ml of freshly prepared DPPH solution (about 40 µg ml^{-1} in MeOH). The absorbance was recorded at 515 nm for one hour, at intervals of 5 min between each reading. Methanol solution (250 µg ml^{-1}) of BHT (butylated-hydroxy-toluene, ALDRICH) was used as positive control. The EC_{50} (concentration of antioxidant required to reduce the initial concentration of free radical DPPH to 50%) was calculated from a calibration curve by linear regression. This methodology for determining the EC_{50} using the computational resource is already properly established.

Antimicrobial activity

The antimicrobial activity of the crude extracts and fractions was determined by the broth microdilution through the minimal inhibitory concentration (MIC). The MIC was defined as the lowest concentration of the extract in which the microorganism did not demonstrate visible growth.

Test microorganisms

Four clinical oral bacteria species from “American Type Culture Collection” (ATCC) were used as test organisms in the screening: aerobic - *Streptococcus sanguinis* (ATCC 10556), *S. mitis* (ATCC 9456), and *S. mutans* (ATCC 25175); anaerobic - *Actonomices naeslundii* (ATCC 19039).

Minimum inhibitory concentration (MIC): broth microdilution method

The assays were carried out under Clinical and Laboratory Standards Institute procedures for aerobic microorganisms (CLSI, 2006) and for anaerobic microorganisms (CLSI, 2007). The MICs of the samples against the test bacterial strains were performed according to the broth microdilution method. The entire procedure was performed in a laminar flow hood, with all of the glassware, pipette tips, culture media and 96-well microplates sterilised (Porto et al., 2009; Carvalho et al., 2011). Samples were dissolved in 1 mg ml^{-1} dimethyl sulphoxide (DMSO), followed by dilution in tryptic soy broth (Difco) for aerobic and Schaedler broth (Difco) supplemented with haemin (5 µg ml^{-1}) and vitamin K1 (10 µg ml^{-1}) for anaerobic; concentrations ranged from 400 to 10 µg ml^{-1}. The final DMSO content was 5% (v/v), and this solution was used as a negative control. The inoculum was adjusted for each organism to yield a cell concentration of 5×10^5 colony forming units (CFU) ml^{-1}. One inoculated well was included to enable control of the adequacy of the broth for organism growth. One non-inoculated well, free of antimicrobial agent, was also employed to ensure medium sterility. Chlorhexidine dihydrochloride (CHD) was used as a positive control. The microplates (96-wells) containing the aerobic microorganisms were sealed with plastic film and incubated at 37°C for 24 h. The anaerobic microorganisms were incubated for 48 to 72 h in an anaerobic chamber (Don Whitley Scientific, Bradford, UK), in 5 to 10% H_2, 10% CO_2, 80 to 85% N_2 atmosphere at 37°C. After that, resazurin (30 µL) in an aqueous solution (0.02% v v^{-1}) was added to the microplates, to indicate microorganism viability for the determination of MIC. Chlorhexidine dihydrochloride (CHD) was used as a positive control and the concentrations ranged from 0.0115 µg ml^{-1} to 5.9 µg ml^{-1}. The controls of sterility of TSB and Schaedler broths, control culture (inoculum), chlorhexidine sterility, sterility of the extracts and control DMSO were all performed and the analyses were performed in triplicate.

Statistical analysis

Determination of ethanolic extracts, total phenolics, proanthocyanidins and antioxidant activity were carried out in triplicate and the results were calculated as mean values (standard deviation). Significant differences were determined by the Tukey test (at a 5% level of significance) for comparison between the extracts and partitions and Holm-Sidak test for comparison between the samples and positive control (BHT), using SigmaPlot vertion 11.0. All data on the biological tests were submitted to treatment ANOVA with a significance level of 5% using the Tukey method in GraphPad Prism 5.

RESULTS

The ethanol extracts were concentrated under reduced pressure, yielding 20.5, 16.4, 14.9, and 0.6 g for leaf, inner bark, outer bark and wood respectively. The liquid-liquid partition of the ethanol extract of the wood was not performed because the mass of extract obtained was insufficient and beyond this fact, the wood consists mainly of lignin and holocellulose. The preliminary qualitative phytochemical tests of the different extracts of *K. coriacea* are shown in Table 1. The extracts showed the presence of terpenoids, saponins, triterpenes, steroids, flavonoids, tannins and phenolic compounds. Alkaloids and amino acids were absent in all extracts.

The results for total phenols, proanthocyanidins content and the effective concentration (EC_{50}) for aerial parts of *K. Coriacea* are presented in Table 2.

The antimicrobial activity of the ethanolic extracts and

Table 1. Preliminary phytochemical screening (qualitative) of different extracts from *K.coriacea*.

Extracts	Phytoconstituents						
	Alkaloids	**Amino acids**	**Terpenoids**	**Saponins**	**Triterpenes and steroids**	**Flavonoids**	**Phenolic compounds and tannins**
EEL	-	-	+	+	+	+	+
CPL	-	-	+	+	+	+	+
DPL	-	-	+	+	+	+	+
EEIB	-	-	+	+	+	+	+
CPIB	-	-	+	+	+	+	+
DPIB	-	-	+	+	+	+	+
MWIB	-	-	+	+	+	+	+
EEOB	-	-	+	+	+	+	+
CPOB	-	-	+	+	+	+	+
DPOB	-	-	+	+	+	+	+
EEW	-	-	+	+	+	+	+

+: Present; -: Absent; EEL: ethanolic extract of leaves; CPL: cyclohexane partition of leaves; DPL: dichloromethane partition of leaves; EEIB: ethanolic extract of inner bark; CPIB: cyclohexane partition of inner bark; DPIB: dichloromethane partition of inner bark; MWIB (9:1): methanol/water partition of inner bark; EEOB: ethanolic extract of outer bark; CPOB: cyclohexane partition of outer bark; DPOB: dichloromethane partition of outer bark; EEW: ethanolic extract of wood.

partitions was screened by the broth microdilution method (BMD) against important microorganisms belonging to strains of the collection ATCC, responsible for human oral diseases. The values of minimal inhibitory concentration (MICs) are presented in Table 3.

DISCUSSION

In general, ethanolic extract and partitions of plant parts of *K. coriacea* showed a very strong antioxidant activity. The inner bark was the plant part that presented the best results. The polar dichloromethane partition presented better results than the cyclohexane partition for total phenols and proanthocyanidns for all plant part analyzed. The outer shell showed the lowest amount of total phenols and proanthocyanidins in this solvent. The lower phenol content and proanthocyanidins found in cyclohexane partitions can be assigned to the non-polarity of the solvent, since the compounds have phenolic hydroxyl and carbonyl groups, which have higher affinity for polar solvents.

The free radical DPPH assay provides basic information on the antiradical activity of extracts (Souza et. al., 2007). According to Table 2, the best results for the antioxidant activity (AA), expressed in terms of average effective concentration (EC_{50}) to scavenge the free radical DPPH, were those found in the inner bark part. Comparing the EC_{50} values with the well-know antioxidant standard BHT, the inner bark extract and partitions showed results similar, exception for the cyclohexane partition.

This part of *K. Coriacea* presented the highest total Phenols and Proanthocyanidins contents in the ethanolic extract and all partitions showing a direct correlation between these bioactive compounds and EC_{50} (Rice-Evans et al., 1997). Besides the antioxidant activity, phenolic compounds present physiologic property as anti-inflammatory and antimicrobial too (Lang and Buchbauer, 2012).

Studies on antimicrobial activity of extracts of others *Kielmeyera* species are known. The ethyl acetate and ethanolic extracts from *K. neglecta* have shown activities against multidrug-resistant bacteria (*E. faecalis* - ATCC 51299 and *S. aureus* - ATCC 43300), with EC_{50} of 12.5 µg ml^{-1} (Adrioli et al., 2012). In this study, the values of minimal inhibitory concentration (MICs) found for aerobic and anaerobic bacteria ranged from 3.2 to 400.

The MIC values determination against oral pathogens using the broth microdilution method has been proved to be useful in the screening of extracts with antimicrobial activity and should be considered as an important tool in researches involving natural products. The ethanolic extract and the cyclohexane fraction of the inner bark were both the most effective against the microorganisms studied. The MICs values for these extracts ranged from 3.1 to 100 µg ml^{-1}. Extracts from plants species with MIC values below 100 µg ml^{-1} and isolated compounds with MICs below 10 µg ml^{-1} are considered promising potential antimicrobial agents (Rios and Recio, 2005). The cyclohexane fraction of the inner bark inhibited the growth of *S. mutans*, the main etiological agent of dental caries at concentrations below 10 µg ml^{-1}.

The ethanolic extract was more effective against the major etiologic agent of dental caries, the aerobic oral

Table 2. Total phenols, proanthocyanidins and EC-50 content in the extracts and partitions from leaves, inner bark (IB), out bark (OB) and wood of *K. coriacea*.

Sample	Total phenols (mg GAE g^{-1} of dry weight of the extract)				Proanthocyanidins (mg CE g^{-1} of dry weight of the extract)				EC_{50} (µg ml^{-1})			
	Leave	IB	OB	Wood	Leave	IB	OB	Wood	Leave	IB	OB	Wood
EE	309.0±4.0^{c}	346.0±2.0^{c}	77.2±2.7^{b}	130.0±2.0	109.0±1.0^{c}	328.0±1.0^{c}	19.9±0.3^{b}	18.4±1.0	10.6±0.7^{a}	5.9±0.1$^{b\#}$	55.2±1.7^{b}	25.5±3.9
CP	57.5±1.0^{a}	82.3±0.6^{a}	27.7±2.7^{a}	-	32.2±1.4^{a}	102.0±1.0^{a}	8.5±0.3^{a}	-	133.0±2.0^{b}	16.0±1.7^{c}	70.1±0.4^{c}	-
DP	139.0±1.0^{b}	241.0±1.0^{b}	125.0±1.0^{c}	-	52.0±2.2^{b}	253.0±3.0^{b}	28.1±1.4^{c}	-	12.7±0.6^{a}	6.6±0.9$^{b\#}$	21.1±0.8^{a}	-
MW (9:1)	-	372.0±1.0^{d}	-	-	-	410.0±3.0^{d}	-	-	-	4.3±0.3^{a}	-	-
BHT*	6.5±0.20$^{\#}$											

EE= ethanolic extract; CP=cyclohexane partition; DP=Dichloromethane partition; MW=methanol/water partition; IB=inner bark; OB=outer bark. Significant differences ($p < 0.05$) are indicated by different letters in each column. Averages followed by the same letter or symbol in the column are not statistically different ($p > 0.05$); *Positive control.

Table 3. Inhibitory effect (MIC values) of the different extracts and partitions from *K. coriacea*.

Sample	MIC (µg ml^{-1})			
	Aerobic			Anaerobic
	S. mitis ATCC 49456	***S. mutans*** ATCC 25175	***S. sanguinis*** ATCC 10556	***A. naeslundii*** ATCC 19039
EEIB	3.1	50	25	100
CPIB	25	6.2	6.2	6.2
DPIB	400	400	>400	>400
MWIB	400	>400	>400	200
EEL	400	>400	400	200
CPL	>400	>400	200	>400
DPL	200	>400	>400	400
EEOB	>400	>400	400	>400
CPOB	400	>400	>400	>400
DPOB	>400	>400	400	400
Chlorhexidine dihydrochloride *	3.688	3.688	1.844	1.844

* Positive control. EEIB: ethanolic extract of inner bark; CPIB: cyclohexane partition of inner bark; DPIB: dichloromethane partition of inner bark; MWIB (9:1): methanol/water partition of inner bark; EEL: ethanolic extract of leaves; CPL: cyclohexane partition of leaves; DPL: dichloromethane partition of leaves; EEOB: ethanolic extract of outer bark; CPOB: cyclohexane partition of outer bark; DPOB: dichloromethane partition of outer bark; EEW: ethanolic extract of wood.

bacteria *S. mitis* (ATCC 49456) (MIC 3.1 µg ml^{-1}), a MIC value lower than the positive control and is below to the isolated substances (Capel et al., 2011), to the same plant part. The cyclohexane fraction was more effective against the aerobic oral bacteria *S. mutans* (ATCC 25175), *S. sanguinis* (ATCC 10566) and against the anaerobic oral bacteria *A. naesllundi* (ATCC 19039) (all with MIC 6.2 µg ml^{-1}).

The ethanolic extract, cyclohexane fraction and dichloromethane fraction of the leaves were more effective against the anaerobic bacteria *A. naesllundi* (ATCC 19039) and against the aerobic bacteria *S. sanguinis* (ATCC 10566) and *S. mitis* (ATCC 49456) respectively, with MIC 200 µg ml^{-1}. These results contribute to the potential use of these extracts against dental caries, supporting results in the folk medicine against dental pain (Neto et al., 2010). On the other hand, the ethanolic extract and partitions of outer bark does not presented the same promising results.

The results reached here can be considered very interesting for most microorganisms tested, below those considered promising for pure substances. In addition, these fractions presented MICs values lower than those found against the same bacteria for extracts and essential oils of others plant from Cerrado biome (Sobral et. al., 2009).

The antimicrobial activity is generally correlated, not only with a class of compounds, but with several of them, acting synergistically. Phenolic compounds present antibacterial activity (Pereira et. al., 2007). These and many others compounds are associated with the antimicrobial activity against dental pathogens (Lang and Buchbauer, 2012). The antibacterial activity of the *K. coriacea* extracts presented here may be related to phenolic compounds, flavonoids and tannins too, in synergism with others metabolic products screened in this work, as indicated by the literature (Wang et. al., 2004; Chang et. al., 2001).

Conclusion

The results of this study showed that the anti-oxidant activity and the antimicrobial activity presented by the ethanolic extract and cyclohexene fraction of the inner bark were considered very promising, for the prevention of dental caries and others oral pathologies. The second highest quantity of phenols and proanthocyanidins contents in this extract may explain these results.

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