Journal of
Pharmacognosy and Phytotherapy

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Journal of Pharmacognosy and Phytotherapy

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Full Length Research Paper

Anticancer activities and safety evaluation of selected Kenyan plant extracts against breast cancer cell lines

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Breast cancer is a leading cause of deaths among women suffering from cancer in Kenya. The current study was done to determine anticancer activities of medicinal plant extracts against breast cancer cell lines (HCC 1395 and 4T1). Vero cells were used for evaluation of safety of extracts. Thiazoly blue tetrazolium bromide (MTT) assay was used in this study. Reference drugs were 5 fluorouracil and cyclophosphamide. Extract concentrations that inhibited growth of cell growth by half (IC$_{50}$) were estimated using GraphPad prism version 7 and 90 % of extracts showed anticancer activities. Methanol extracts of *Uvariodendron anisatum*, *Fagaropsis angolensis*, *Combretum tanaense*, *Hydnora abyssinica* and water extract of *F. angolensis* exhibited remarkable anticancer activities (IC$_{50}$< 30 μg/ml). Methanol extracts of *F. angolensis* and *H. abyssinica* demonstrated high selectivity index (SI ≥3). Evaluation for safety, indicated that about 64% of the extracts under this study were non-toxic (CC$_{50}$ >100 μg/ml). Findings from plants in this study support folklore claims. Phytochemical analysis, bioassay guided fractionation and toxicity studies are underway on extracts of *C. tanaense, F. angolensis, H. abyssinica* and *U. anisatum*.

Key words: 4TI, ethnomedicine, HCC 1395, IC$_{50}$ values, medicinal plants, MTT assay, selectivity index, vero E6.

INTRODUCTION

Breast cancer is the most frequently diagnosed and leading cause of cancer deaths among women. It caused about 522,000 deaths in 2012 worldwide and these estimations are expected to double by the year 2030(WHO, 2014). Of all reported cases of cancer in Kenya, breast cancer has a prevalence of 23.3% and is

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the most common among women (Ministry of Public Health and Sanitation and Ministry of Medical Services, 2011-2016). The reduced access to comprehensive cancer care services, high cost of health services and inadequate cancer specialized health personnel further aggravate the cancer burden. Increasing burden of breast cancer cases in Kenya calls for a number of interventions (Policy Brief, 2011).

Furthermore, as a commitment towards realization of vision 2030 in Kenya, policies that address prevention and management of cancers are being implemented. Kenya is also making every effort to reduce pre-mature mortality from non-communicable diseases (NCDs) by one-third as a commitment towards the third goal of sustainable development (ICSU, ISSC, 2015, United Nations (UN), 2015). It is estimated that 80% of world population use traditional medicine (WHO, 2005; Malki, 2013). In connection with traditional medical systems, about 91% of cancer patients seek complementary alternative medicine (CAM) services worldwide and up to 98% of these patients are those suffering from breast cancer (Leung and Fong, 2007; Mazzio and Soliman, 2009). In other regions of the world, CAM has been useful in the discovery and development of drugs and drug derivatives which are useful clinically in the management of breast cancer. Two of these plants used for more than a century now are *Catharanthus roseus* (first identified in1950s) as a source of vincristine and vinblastine and *Taxux brevifolia* is a known source of taxol since 1962 (Evans, 2009). On the other hand, elliptinium isolated from *Bleekeria vitensis* has also been prescribed for breast cancer treatment for more than ten years now (Shoeb, 2006).

Continued search for plant compounds or products is necessary, for discovery of lead compounds with antitumor activities for breast cancer. Moreover, traditional medicine practices employ plants in treatment, prevention or management of breast cancer (King Saud University, 2016; Prakash et al., 2013). In Kenya, up to 70% of the over 43 million people use traditional medicine in primary health care. Cancer patients use plant based medicines to complement or as alternatives for conventional medicines (Njoroge and Kibunga, 2007). A number of plants are used ethnomedically in the management of breast cancer and some have been documented (Kareru et al., 2007; Ochwangi et al 2014). The plant extracts under this study: *Combretum tanaense* (Combretaceae), *Fagaropsis angolensis* (Rutaceae), *Hydnora abyssinica* (Hydnoraceae), *Launaea cornuta* (Asteraceae), *Prunus* and *Uvariodendron anisatum* (Annoanceae) have been *africana* (Rosaceae), *Spermacoce princeae* (Rubiaceae) reported in the literature to manage cancer (Kareru et al., 2007; Kokwaro, 2009; Jeruto, et al., 2008, 2011; Kigen et al., 2013; Ndwigah et al., 2014).

The objective of the current study was to establish anticancer activities of selected plant extracts using HCC1395 and 4T1 breast cancer cell lines.

MATERIALS AND METHODS

Five of the plants were obtained from Embu County (roots of *U. anisatum* from Kiangombe forest, rhizomes of *H. abyssinica* from Ishiara Karuri village, barks of *F. angolensis*, *P. africana* and aerial parts of *L. cornuta* from Irangi forest in Embu). The roots of *C. tanaense* were collected from Mount Kenya University botanical garden in Thika County, while the aerial parts of *S. princeae* were collected from Mabariri village, Bomwagamo location in Nyamira County. The collected specimens were identified and authenticated with the aid of a taxonomist at the National Museums of Kenya (East Africa Herbarium) where the voucher specimens were prepared and deposited. The plant voucher specimens were as provided in the parentheses, *U. anisatum* (JMO-1-2015), *Hydnora abyssinica* (JMO-2-2014), *F. angolensis* (JMO-3-2015), *P. africana* (JMO-3-2014), *L. cornuta* (JMO-1-2014), *C. tanaense* (JMO-2-2015) and *S. princeae* (JMO-4-2015).

Extraction and preparation of test extracts

The collected plant samples were air-dried under shade and thereafter they were ground using an electric mill. Methanol extracts were prepared by cold maceration for 48 h, and 250 g of the powders were soaked in 2.5 conical flasks using methanol (1 L). The methanol extracts were filtered and concentrated *in vacuo* at 50°C and finally dried in an oven at 35°C. Water extracts were obtained by boiling 50 g of the powdered drug in distilled water (0.5 L) for 5 min, the water extracts was then allowed to cool, filtered and then freeze dried. The dry extracts were weighed and stored in a freezer at -20°C. Stock solutions (10 mg/ml) of all extracts were made for anticancer assay, 10 mg of the dry extracts were dissolved in 100 µl of dimethylsulfoxide (DMSO) and then added up to 1000 µl with phosphate buffer solution (PBS). The stock solutions for each extract were then serially diluted by using PBS to obtain working concentrations ranging from 1000 to 0 µg/ml. The preparations were done under sterile conditions and the solutions of extracts were stored at 4°C until use.

Cancer cell lines and cell culture preparations

Human breast cancer cell line (HCC 1395), mouse breast cancer cell line (4TI) and normal kidney epithelial cells from African green monkey (Vero E6) cell line were obtained from the American Type Culture Collection (ATCC) (Rockville, USA). HCC 1395 (ATCC® CRL-2324™) cells were cultured and maintained using Roswell Park Memorial Institute (RPMI-1640). Normal kidney epithelial cells from African green monkey (Vero E6) and 4T1 cells were cultured and maintained using Eagle's Minimum Essential Medium (EMEM). All cell cultures were supplemented with 100 units/ml penicillin/streptomycin and 10% fetal bovine serum (FBS) and they were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Anticancer activities assay of crude extracts

Standard 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell viability in the presence and absence of extract(s). The cells were plated in 96-well plates at a density of approximately 2 x10^4 cells per well and suspended in 100 µl of media. The plates were then incubated for 24 h at 37°C, 5% CO_2 and relative humidity of 95% to attach. Thereafter, the extracts and standard drugs were added to the wells at concentrations ranging from 1000 to 0 µg/ml. The experiment was designed in such a manner that experimental blanks (wells containing media and test drug) and negative control (wells containing media and cells) were run simultaneously in triplicates.

Table 1. IC_{50} values (µg/ml) of extracts against HCC 1395 breast cancer cell line.

Plant	Extract	Cell lines		SI
		HCC 1395	**Vero**	
		IC_{50} (µg/ml)	CC_{50} (µg/ml)	
Uvariodendron anisatum root	Methanol	50.6±2.9	3.3±0.2	0.06
	Water	248.0±5.8	153.5±1.5	0.6
Hydnora abyssinica rhizome	Methanol	27.20±1.1	84.23±6.3	3.10
	Water	499.3±1.3	184.00±12.00	0.37
Launaea cornuta leaf	Methanol	231.7±2.0	384.00±32.5	1.7
	Water	365.0±15.3	>1000	>2.7
Combretum tanaense root	Methanol	193.0±13.2	36.16±4.0	0.19
	Water	>1000	>1000	>1
Fagaropsis angolensis bark	Methanol	59.4±5.6	21.7±6.6	0.36
	Water	553.6±15.4	302.7±16.6	0.5
Spermacoce princeae aerial	Methanol	533.00±56.6	203.00±4.9	0.4
	Water	940.33±53.3	576.00±36.7	0.6
Prunus africana bark	Methanol	10.6±0.7	20.5±0.6	1.93
	Water	81.9±8.04	196.00±6.00	2.39
5-Fluorouracil (positive control)		38.8±7.56	185.00±75.0	4.79
Cyclophosphamide (positive control)		32.8±1.1	2.78±1.1	0.08

Data is presented as mean±SEM of IC_{50} (µg/ml) from three independent experiments.

The plates were once again incubated for 48 h at conditions described above. MTT reagent (10 µl) was added to each well and the plates were further incubated for 4 h after which the supernatant was aspirated. Pure DMSO (100 µl) was added to each well to solubilize MTT crystals. The plates were then read for colour absorbance on an ELISA scanning multiwell spectrophotometer (Multiskan Ex labsystems) at 562 nm. The standard drugs, 5-fluorouracil and cyclophosphamide were used as positive controls. Percentage cell cytotoxicity was calculated using the formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Absorbance of cells without treatment} - \text{Absorbance of cells with treatment}}{\text{Absorbance of cells without treatment}} \times 100$$

Selectivity index (SI) which indicates the ability of the drug to discriminate against cancerous cell and in favour of normal cells was calculated using the following formula:

$$\text{SI} = \frac{CC_{50} \text{ Value for Vero cells}}{IC_{50} \text{ Values for Cancer cells}}$$

Where, CC_{50} is the concentration of the extracts that exerted cytotoxic effects on half of the population of normal cells and IC_{50} is the concentration of the extracts that inhibited growth/proliferation of half of the population of cancerous cells. All the cytotoxicity procedures were performed at Kenya Medical Research Institute (KEMRI) after the approval of the institutional Scientific and Ethics Review Unit.

Data analysis

Raw data for MTT assay was transfered for processing using Microsoft Excel 2013 to compute optical densities and IC_{50}. Statistical analysis was performed with GraphPad Prism Version 7 to express the IC_{50} of the three independent experiments as mean ± standard error of the mean (SEM). Anticancer activities of the crude extracts was analyzed with respect to the American National Cancer Institute criteria where crude extracts that had IC_{50}< 50 µg/ml were considered active and those with IC_{50}< 30 µg/ml as remarkably active (Boik, 2001; Wratchanee et al., 2010; Siti et al., 2011). The safety levels of the extracts were estimated by determination of CC_{50} values; extracts that had CC_{50}>100 µg/ml were considered safe. The SI values were calculated and selectivity was denoted as high, moderate or non-selective where the values were ≥3, between 1 and 3, and <1, respectively.

RESULTS

Anticancer activities of extracts against HCC 1395 breast cancer cell line

Investigations of anticancer activities of plant extract against human breast cancer cell line (HCC 1395) gave results as indicated in Table 1. Methanol extracts of the

Table 2. IC_{50} values (µg/ml) of extracts against 4T1 breast cancer cell line.

| Plant | Extract | Cell lines | | SI |
| | | 4T1 | Vero | |
		IC_{50} (µg/ml)	CC_{50} (µg/ml)	
Uvariodendron anisatum root	Methanol	1.77±0.06	3.3±0.2	1.86
	Water	150.7±4.9	153.5±1.5	1.02
Hydnora abyssinica rhizome	Methanol	22.9±0.1	84.23±6.3	3.68
	Water	79.8±1.0	184.00±12.00	2.3
Launaea cornuta leaf	Methanol	300.5±5.5	384.00±32.5	1.28
	Water	700.5±14.5	>1000	>1.4
Combretum tanaense root	Methanol	19.5±0.00	36.16±4.0	1.9
	Water	289.7±2.9	>1000	>3.45
Fagaropsis angolensis bark	Methanol	12.9±1.2	21.7±6.6	1.68
	Water	80.0±1.7	302.7±16.6	3.78
Spermacoce princeae aerial	Methanol	204.00±6.6	203.00±4.9	1.00
	Water	562.00±10.00	576.00±36.7	1.00
Prunus africana bark	Methanol	4.78±0.96	20.5±0.6	4.30
	Water	36.77±8.56	196.00±6.00	5.33
5-Fluorouracil (positive control)		>1000	185.00±75.0	0.19
Cyclophosphamide (positive control)		>1000	2.78±1.1	0.002

Data is presented as mean±SEM of IC_{50} (µg/ml) from three independent experiments.

H. abyssinica rhizome and *P. africana* bark were the most promising with estimates of IC_{50} values being 27.20±1.1 and 10.6±0.7 µg/ml, respectively. Other extracts that had high activity were *U. anisatum* root methanol, *F. angolensis* bark methanol and *P. africana* bark water (IC_{50}= 50.6±2.9, 59.4±5.6 and 81.9±8.04 µg/ml, respectively). Nine extracts exhibited low anticancer activities against HCC 1395 cell line (100 ≤IC_{50}≤1000 µg/ml) as shown in Table 1 and *C. tanaense* water extract was considered inactive (IC_{50}>1000 µg/ml).

The results reveal that two methanolic plant extracts were more potent against HCC 1395 breast cancer cell line as compared to the reference drugs (cyclophosphamide and 5 fluorouracil). *H. abyssinica* rhizome (IC_{50}=27.20±1.1 µg/ml) and *P. africana* bark (IC_{50}=0.6±0.7) were more active against HCC1395 human breast cancer cell line. The IC_{50} for 5-fluorouracil was 32.8±1.1 and that of cyclophosphamide was 38.8±7.56 µg/ml against the same breast cancer cell line.

Anticancer activities of extracts against 4T1 breast cancer cell line

Five extracts demonstrated remarkable activities against mouse breast cancer cell line (4T1); they had IC_{50} estimates at concentrations below 30 µg/ml and these extracts were all methanolic extracts of *U. anisatum* root, *H. abyssinica* rhizome, *C. tanaense* root, *P. africana* bark and *F. angolensis* bark. The water extracts obtained from *H. abyssinica* rhizome, *P. africana* bark and *F. angolensis* bark had activities at concentrations 30≤IC_{50}≤100 µg/ml and was regarded to have high activity. All the other remaining six extracts under this study demonstrated low activity as indicated in Table 2.

The IC_{50} values for the active extracts against 4T1 cell lines, *U. anisatum* root methanolic extract (IC_{50}=1.77±0.06 µg/ml), *P. africana* bark methanolic extract (IC_{50}=4.78±0.96 µg/ml), *F. angolensis* bark methanolic extract (IC_{50}=12.9±1.2 µg/ml), *C. tanaense* (IC_{50}=19.5±0.0 µg/ml) and *H. abyssinica* rhizome methanolic extract (IC_{50}=22.9±0.1 µg/ml) are shown. Interestingly, it was observed that the standard reference drugs (5-fluorouracil and cyclophosphamide) were inactive against 4T1 breast cancer cell line.

Safety of extracts against vero cell line

Nine out of the 14 extracts exhibited CC_{50} values which were greater than 100 µg/ml; these included, methanoland water extracts obtained from *L. cornuta*

leaves and *S. princeae* aerial part; water extracts of *U. anisatum* root, *H. abyssinica* rhizome, *F. angolensis* bark, *C. tanaense* root and *P. africana* bark. Most of the methanol extracts of plants whose water extracts have been depicted as safe were found to be toxic against vero cells; they demonstrated CC_{50} values ranging from 3.3±0.2 to 84.23 ±6.3 µg/ml as indicated in Table 2. *U. anisatum* root methanol extract was found to be highly toxic; it demonstrated the lowest CC_{50} values against vero cell line (CC_{50}=3.3±0.2 µg/ml).

Methanol extracts of *P. africana* bark (CC_{50}=20.5±0.6 µg/ml), *F. angolensis* bark (CC_{50}=21.7 ±6.6 µg/ml) *C. tanaense* root (CC_{50}=36.16±4.0 µg/ml) and *H. abyssinica* rhizome (CC_{50}=84.23±6.3 µg/ml) were all considered toxic since they had CC_{50} values at concentrations that were less than 100 µg/ml. The standard reference drugs, 5-fluorouracil and cyclophosphamide had varied toxicity levels against the vero cell line; 5-fluorouracil (CC_{50}>100 µg/ml) was considered nontoxic, while cyclophosphamide was found to be toxic with CC_{50} values estimated at 2.78±1.1 µg/ml (Table 1).

Selectivity index (SI)

The calculation of selectivity index (SI=$\frac{CC50}{IC50}$), was used to establish the ability of the extracts to discriminate their effect against normal and cancer cell lines. The calculated SI values are indicated in Tables 1 and 2 for HCC 1395 and 4T1 breast cancer cell lines, respectively. Two methanol extracts, *H. abyssinica* rhizome (SI=3.10) and *P. africana* bark (SI=1.93) demonstrated moderate selectivity index following their effect on HCC 1395 breast cancer cell line. All other extracts under this study were non selective in their activity in relation to HCC 1395 breast cancer cell line. *L. cornuta* leaf water extract was the only water extract that was observed to have high selectivity index (SI>2.7).

However, it had low activity against HCC 1305 cell line. The standard reference drugs demonstrated varying SI values with 5-fluorouracil having high selectivity (SI=4.79), whereas cyclophosphamide was non selective; it had SI values of 0.08 in relation to HCC 1395 breast cancer cell line as shown in Table 1.

Higher selectivity indices were recorded on 4T1 breast cancer cell line, water extracts of *F. angolensis* bark (SI=3.78) and *P. angolensis* bark (SI=5.33). Methanol extracts also had high activity on this same cell line, *H. abyssinica* rhizome (SI=3.68) and *P. africana* bark (S=4.3) as shown in Table 2. All the other extracts exhibited moderate selectivity indices with values ranging from 1.00 to 2.3, it is also noted that SI values for water extract of *C. tanaense* root were high (SI>3.45) on 4T1 breast cancer cell line. The SI values for the standard reference drugs as indicated in Table 2 shows that both 5-fluorouracil and cyclophosphamide were non-selective in the case of 4T1 breast cancer cell line.

DISCUSSION

The strength of anticancer activities of the extracts in this study varied in relation to extracts and breast cancer cell line; extracts of *H. abyssinica* rhizome demonstrated anticancer activities against HCC 1395 and 4T1 breast cancer cell line. A study conducted by Yagi et al. (2012) on cytotoxicity of water and 70% ethanol extracts of *Hydnora johannis* root, showed that the extracts had anticancer activity against human mouth epidermoid carcinoma (KB) cell line. Low toxicity of *H. abyssinica* rhizome extracts was reported against vero cell line; this is in agreement with the study done by Koko et al. (2009) and Yagi et al. (2012) which establish the safety of 80% ethanol extract of *H. abyssinica* against 3T3 mouse fibroblast cell line; 70% ethanol extract of *H. johannis* root against MRC5 (derived from non-cancer human fetal lung), respectively. Other toxicity studies that were done by Osman (2010) using rats, showed that water extract of *H. abyssinica* was non-toxic at dose of 1600 mg/kg and the safety studies are consistent with the current studies.

P. africana bark extracts were found to be active against HCC 1395 and 4T1 breast cancer cell line; anticancer activity against HCC 1395 breast cancer cell line is reported for the first time in the current study. Studies done by Nabende et al. (2015) using 4T1 breast cancer cell line showed that methanol extract of *P. africana* bark was active while the water extract was inactive. Contrastingly, in the current study, water extract was found to be active against 4T1 breast cancer cell line. In other anticancer studies, ethanolic extract of *P. africana* bark was found to have significant anticancer activities against PC3 and LNCaP human prostate cancer cell lines with IC_{50} values estimated at about 2.5 µl/ml (Shenouda et al., 2007). The water extract of *P. africana* bark is reported to be safe in this study; this finding is consistent with that of Karani et al. (2013), where estimated CC_{50} values of *P. africana* bark were 104.08 µg/ml. It is known that extracts with CC_{50} of more than 100 µg/ml are considered to be safe in cytotoxic studies.

The anticancer activities of the remaining five plants in the current study: *F. angolensis*, *C. tanaense*, *U. anisatum*, *L. cornuta* and *S. princeae* has not been previously reported. Methanol extracts of two plants: *F. angolensis* bark and *U. anisatum* root exhibited considerable anticancer activities on breast cancer cell lines (4T1 and HCC 1395). *C. tanaense* root methanol extracts were active against 4T1 and inactive against HCC 1395 breast cancer cell lines. Except for water extracts of *F. angolensis* which had high activity, all the other water extracts demonstrated low activities against the anticancer cell lines. The activities of methanol extracts of *L. cornuta* and *S. princeae* were also reported to be low.

The variations in anticancer activities that was observed in different extracts, indicate that methanol was a better solvent for extracting compounds with anticancer

activities. Most of the extracts in this study were non-toxic except for methanol extracts of *U. anisatum* root which was rated highly toxic, followed by *F. angolensis* bark, *H. abyssinica* rhizome and lastly *C. tanaense* root. It was established that the extracts that had high activities against cancer cell lines were toxic to the normal cell lines. The active extracts, *H. abyssinica* rhizome, *U. anisatum* root, *P. Africana* bark, *F. angolensis* bark and *C. tanaense* root exhibit differential selectivity and therefore demonstrated ability to distinguish between cancer and normal cell lines in this study. Previous studies done by Nabende et al. (2015), established that methanol extract of *P. africana* bark distinguished normal vero cell line from 4T1 breast cancer and CT26 human colon cancer cell lines by SI values of 7.26 and 1.11, respectively, this means that the methanol extract of *P. africana* bark had high cytotoxic effect against cancer cell line, on the other hand, it has low toxicity against normal cell line.

Conclusion

The findings of this study provide a scientific justification for the traditional use of these plants. The anticancer activity of 90% of these plants against 4T1 and HCC 1395 breast cancer cell lines is being reported for the first time in the current study. Methanolic extracts of *C. tanaense* (root), *P. africana* (bark), *H. abyssinica* (rhizome), *F. angolensis* (bark) and *U. anisatum* (root) possess high anticancer activities. Out of the five extracts with high potency, the methanol extract of *H. abyssinica* rhizome was considered the safest followed by methanol extracts of *C. tanaense* root, *F. angolensis* bark, *P. africana* bark and *U. anisatum* root in decreasing order of safety. Presently, these extracts are being investigated by bioassay guided fraction to establish the compounds that are responsible for activity against breast cancer cell lines.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

REFERENCES

Boik J (2001). Natural Compounds in Cancer Therapy. Oregon Medical Press, Minnesota, USA.

Evans WC (2009). Trease and Evans' Pharmacognosy. (15th Edition), London: WB Saunders Company Ltd.

Jasneef A, Swatantra BS, Shivshanker P (2013). Phytochemical screening and physicochemical parametersof crude drugs: A brief review. Int. J. Pharm. Rev. 2(12):53-60.

Jeruto P, Mutai C, Catherine L, Ouma G (2011). Phytochemical constituents of some medicinal plants used by the Nandis of South Nandi district. J. Anim. Plant Sci. 9(3):1201-1210.

Jeruto P, Mutai C, Catherine L, Ouma G, Otieno D (2008). An ethnobotanical study of medicinal plants used by the Nandi people in Kenya. J. Ethno. Pharmacol. 116(1):370-376.

Karani LW, Tolo FM, Karanja SM, Khayeka- Wandabwa C (1023). Safety of *Prunus africana* and *Warbugia ugandensis* in asthma treatment. South African Journal of Botany 88:183-190

Kareru PG, Kenji GM, Gachanja AN, Keriko JM, Mungai G (2007). Traditional medicines among the Embu and Mbeere people of Kenya. Afr. J. Tradit. Complement Altern. Med. 4(1):75-86.

Kigen KG, Ronoh KH, Kipkore WK, Rotich KJ (2013). Current trends of traditional herbal medicine practice in Kenya: A Rev. 2(1):32-37.

Kokwaro JO (2009). Medicinal plants of East Africa (Third edition) Kenya: East Africa Literature Bureau.

King Saud University (2016). Natural cures for breast cancer treatment. Saudi Pharmaceutical Journal 24:233-240

Leung PC, Fong H (2007). Alternative Medicine for Cancer. Ann. Tradit. China Med. Vol. 3. World Scientific Publishing Co. Pte. Ltd.

Malki AM (2013). Cancer treatment strategies. OMICS Group eBooks.

Mazzio AE, Soliman KFA (2000). *In Vitro* screening for tumoricidal properties of International Medicinal Herbs. J. Phytother. Res. 23(3):385-398.

Ministry of Public Health and Sanitation and Ministry of Medical Services (2011-2016). National Cancer Strategy 2011-2016. Republic of Kenya.

Nabende PN, Karanja SM, Mwatha JK, Wachira SW (2015). Anti-proliferative activity of *Prunus africana*, *Warbugia stuhlmanni* and *Maytenus senegalensis* extracts in breast and colon cancer cell lines. Eur. J. Med. Plant 5(4):366-376.

Ndwigah N, Stanely N, Amugune K, Beatrice K, Thoithi N, Grace N, Mwangi W, Mwangi W, Mugo N, Hannington N, Kibwage O (2014). Antibacterial and antifungal activity of *Dombeya torrida* (JF Gmel) and Hydnora abyssinica (A. Braun). Afr. J. Pharmcol. Therap. 3(1):14-18.

Njoroge GN, Kibunga JW (2007). Herbal medicine acceptance, sources and utilization for diarrhoea management in a cosmopolitan urban area (Thika, Kenya). Afr. J. Ecol. 45(1):65-70.

Policy Brief (2011). On the situational analysis of cancer in Kenya (2011). Republic of Kenya: National Assembly.

Siti SMM, Nurhanan MY, Muhd Haffiz J, Mohd Ilham A, Getha K, Asiah O, Norhayati I, Lili Sahira H, Anee S (2011). Potential anticancer compound from Cebera Odollam. J. Trop. Forest. Sci. 23(1):89-96.

World Health Organization (WHO) (2014). Global status report on non-communicable diseases. Geneva. ISBN: 9789241564854. pp. 154-181.

Wratchanee M, Vithoon V, Wanna C, Arunporn I, Kesara NB (2010). Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in vitro*, BCM Compliment. Altern. Med. 10(55):1-8.

Full Length Research Paper

Pharmacognosy and phytochemical standardization of *Albizia Ferruginea* (Guill and Perr) pulps

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In Africa and Cameroon in particular, people tend to use medicinal plants as the first approach to cure their illness or disease. In order to improve the safety and the progressive integration of those plants in our health systems, it is important to set a system and protocol that would permit an easy characterization, quality and safety control of medicinal plants through a standardization of their recipes. Thus, we develop a Pharmacognosy and phytochemical study of *Albizia ferruginea* pulps and complete by a monograph for each plant. A macroscopic and microscopic analysis of the drugs is done, followed by a study of the weight loss due to desiccation. Furthermore, the ratio of ashes in the powders was investigated in conformity with the European pharmacopeia. We further extract and perform a phytochemical screening. From the macroscopic analysis, it is observed that the pulp was strong, fibrous crack and powder brown with an aromatic smell. The micrographic analysis shows the presence of various tissues such as suber, fiber, liber, sclerotic cells as well as the calcium oxalate. The ashes percentage of *A. ferruginea* powder is 1.16%, whereas the weight loss due to desiccation is 7.6 % and inflation indices, 4 mL. The phytochemical screening showed the presence of alkaloids, flavonoid, polyphenols, mucilages, anthocyanins, and saponins. The information gathered throughout this study will contribute to the fast identification, quality control, and characterization of *A. ferruginea* pulps.

Key words: Standardisation, pulps, quality control, *Albizia ferruginea*.

INTRODUCTION

According to the World Health Organisation (WHO), infectious diseases are causes for more than 17 million

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death per year and causes for more than half the dead casualty occurring in Africa (Organisation Mondiale de la Sant´e, 2006). Those infectious diseases are rapidly spraying and causing more skin conditions in the continent.

Due to the low level of income in Cameroon and the high cost of curing skin conditions, it is necessary to investigate new methods, substances, plants at low cost that could be used to efficiently cure certain types of skin conditions. In order to achieve that goal, it is important to do further investigation into the field of medicinal plants (Kouadio et al., 2015). It is reported that up to 80% of African regularly use traditional medicine (Observatoire de la sant´e en Afrique, M´edecine traditionnelle, Revue du bureau r´egional de l'OMS pour l'Afrique, 2003).

Traditional medicine is widely used in Africa, due to the wide availability of plants, and their low cost. Therefore it is estimated that we have a ratio of one traditional healer per 500 patient compare to one medical doctor for 40,000 patients in average (Organisation Mondiale de la Sant´e, 2006, 2014). Strat´egie de l'OMS pour la m´edecine traditionnelle pour 2014-2023). Abondo-Ngono et al. (2015) reported in 2012 on a census of traditional healers in the Centre region of Cameroon. Hence they launched the mapping of Cameroonian traditional healers (Abondo-Ngono et al., 2015).

Subsequently, Abondo-Ngongo et al. (2015) have chosen a certain traditional recipe that is actively used to cure ringworms. The recipe contains a medicinal plant locally called "Evouvou" in Ewondo language, scientifically known as *Albizia ferruginea* (Abbiw, 1990) (Figure 1 and 2). This study seek to improve the quality of the recipe commonly used by traditional healers, in order to make these process much valuable and safe for the patients. This work consists of making a data base and specification of medical materials, in order to build a monography data base for the plant and the recipe. Therefore, the standardization reference of the powdered pulps of *A. ferruginea* was used.

MATERIALS AND METHODS

Vegetal material

The vegetal material consists of *A. ferruginea* pulps harvested on February 1, 2016, at Eloundem, located at almost 10 km from the Yaounde city center (add the GIS location of the site). Following the harvesting, a systematic identification of the specimens was made at the Cameroonian herbal data center, then a comparison with the 49871/HNC specimen was made for the *A. ferruginea*.

Preparation of hydro-alcholic extracts

The pulps were dry for a week in an ambient environment, followed by a grinding to a granular powdered form. A sample of the powder can be used for macroscopic characterization using the conventional   method recommended by the World Health Organization (1998). The microscopic characterization allows us to determine the quality of the medicinal plant. Another sample was used for microscopic study following the process described by Blond et al. (2014). A powder mass of 250 g was used for the extract, using a Soxhlet from BEHR LABOR-TECHNIK. Therefore, a mixture of ethanol-water in a volume ratio of 70/30   was used as a solvent. The extract obtained from this extraction was used as phytochemical screening.

RESULTS

Microscopic characterization

Investigation of foreign elements

A powder of mass 400 g was sprayed on a thin film according to the European Pharmacopeia technique (2008). Throughout this characterization, no foreign element was found nor identified.

Powder description

The color was brown with an aromatic odor.

Microscopic analysis

The microscopic analysis showed the crystal of Calcium Oxalate, the fibers in pericyclic forms, the wooden vessel in the spiral forms, the clusters of sclerotic cells, and the suber (Figures 3, 4, 5). The observation wasoptimal with potash (M´ethodes de pharmacognosie,Pharmacop´ee europ´eenne 6`eme ´edition, 2008; P´elissier Y. Travaux pratiques de pharmacognosie micrographie, 2012).

Phytochemical analysis

The loss due to the desiccation of the vegetal drugs, as well as their content in ashes, was evaluated using the 6th edition of the European Pharmacopeia (M´ethodes de   pharmacognosie,Pharmacop´ee   europ´eenne 6`eme ´edition, 2008). We obtained 1.16% in content of total ashes, and 7.6% in term of lost due to desiccation.

Inflation indices

This was determined using the Guedouari (2011; 2012) method, and was found to be 4 mL.

Phytochemical screening

Investigation of flavonoids

Using the method developed in Alzoreky and Nakahara (2003), 2 mL of a solution of soda was added to 2 mL of extract led to an intense yellow coloration.

Investigation of alkaloids

Using the method proposed by Bagre et al. (2007), 0.5 g

Figure 1. Image of *Albizia ferrugiena* pulps (Left: outter surface, Right: inner surface).

Figure 2. Photograph showing the powder Albizia ferrugiena pulps).

of extract was mixed with 0.5 mL of aqueous chlorhydric acid in a steaming bath. After an addition of 3 to 5 drops of a solution of picric acid, we obtained turbidity.

Investigation of polyphenols

Using the method of Bagre et al. (2007), a test tube was used to dissolve 0.1 g of vegetal extract in a mixture of ethanol- water. After adding a drop of a 2% of an alcoholic solution of iron chloride, we observed a change of coloration which changes to a greenish yellow.

Investigation of coumarins

Using the method described in Alzoreky and Nakahara (2003), a test tube is used to dissolve 0.1 g of extract in 2

mL of distilled water. The addition of 2 mL of sodium Hydroxide led to an orange coloration.

Investigation of anthocyanins

Using the method described in Alzoreky and Nakahara (2003), a test tube was used to dissolve a 0.1 g extract in 2 mL of distilled water. The addition of 2 mL of sulfuric acid led to the apparition of red-orangish which quickly turned to a purple blue, using the protocol of Ayoola et al. (2008).

Investigation of saponins

5 mL of distilled water was used to dissolve 5 mg of extract. We observed the apparition of an important foam with a
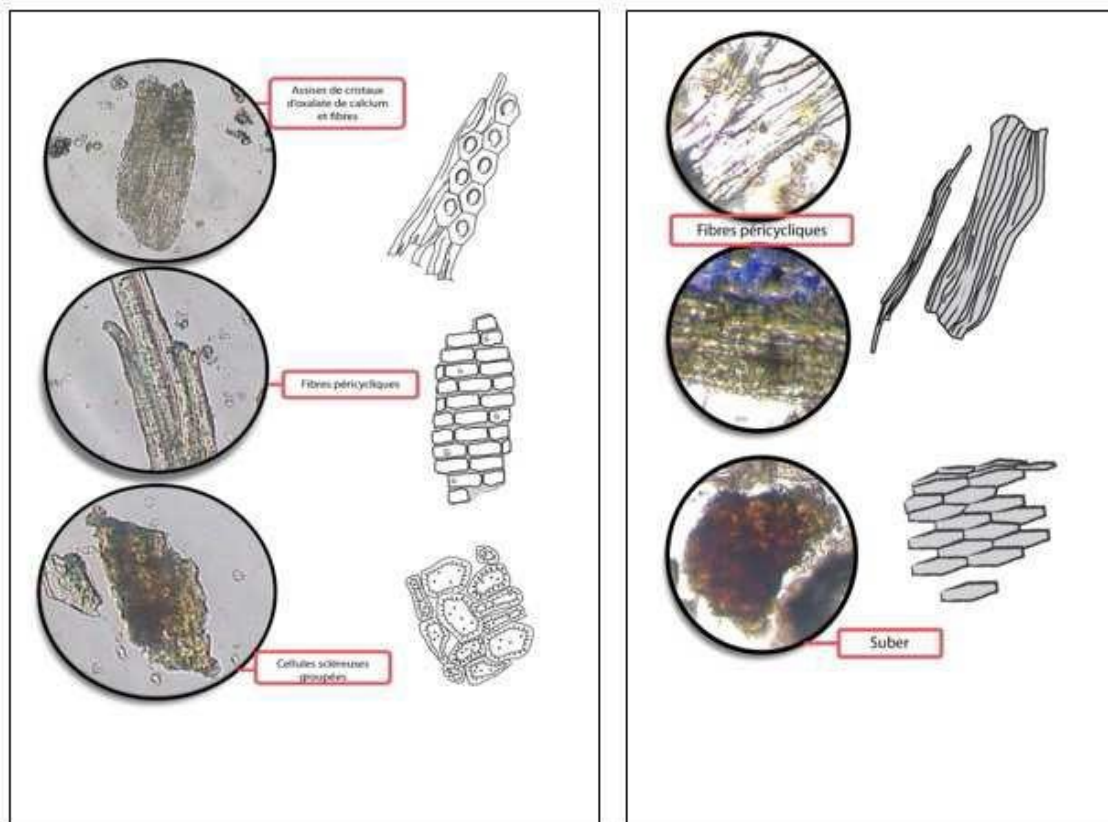
Figure 3. (A). Micrographs showing the crystal of calcium oxalate. From top to the bottom we have the fibers, the pericyclic fibers and the clustered sclerotic cells) (B).Micrographs showing the pericyclic fibers (top), and the suber (down).

Figure 4. Micrograph showing spiral wood vessels.

Albizia ferruginea

DEFINITION :

Dry pulps: division of the *Albizia ferruginea (Guill & Perr)*

Harvested in February 2016

CHARACTERS

Organoleptic characters: Brown color on the outter surface and white in the inner surface

IDENTIFICATION

Botanical macroscopic character: Thick pulps with small and rough lamella and a red

sticky exudate.

Botanical microscopic character: See illustration below

TEST:

Total ashes: 1.16 %

No presence of foreign element
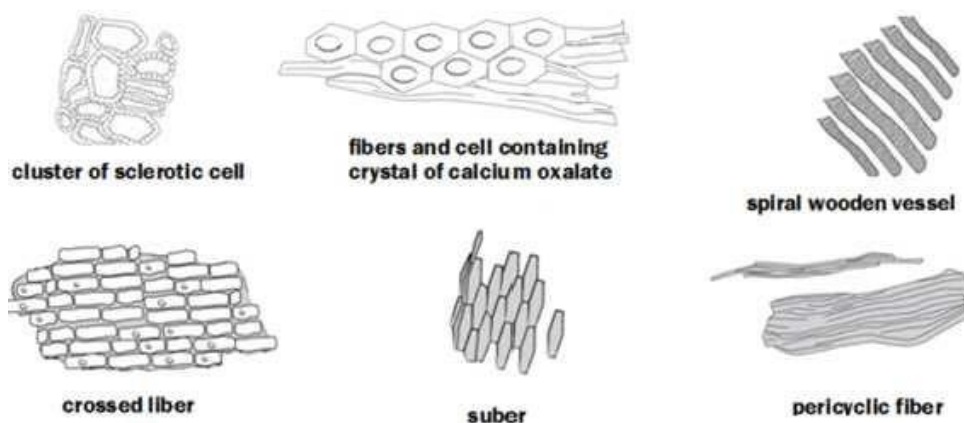
Lost due to dessication: 7.6%

Inflation indices: 4 mL

Aqueous extraction with a soxhlet: Alkaloids, polyphenols, mucilages, flavonoids, anthocyanins, saponoside

Hydro-alcoholic extraction with a soxhlet: Alacaloids, polyphenols, mucilages, flavonoids, anthocyanins,

Conservation: Far from light in a shaded environment

cluster of sclerotic cell

fibers and cell containing
crystal of calcium oxalate

spiral wooden vessel

crossed liber

suber

pericyclic fiber

Figure 5. Micrograph showing spiral wood vessels.

thickness greater than a centimeter.

Investigation of terpenes

Pursuing with the protocol of Ayoola et al. (2008), 0.5 g of extract was mixed with 2 mL of chloroform. After adding 3 mL of a solution of sulfuric acid, we observed an apparition of a ring with a brown-red coloration.

Investigation of "tannins"

Still using the approach from Ayoola et al. (2008), 10 mL of

distilled water was added to 0.5 g of extracts. The addition of few drops of an iron chloride led to a brown coloration.

Investigation of mucilage

Following the protocol of Abayomi (2010), 2 mL of extracts was added to 4 mL of alcohol at 90°C. We observed a rising of air bubbles.

Investigation of steroids

Following the protocol of Surendra et al. (2013), 2

Table 1. Macroscopic pulps of *Albizia ferrugiena*.

Pulps	Surface	Fracture	Pulps aspect
Albizia ferruginea	Hard	Fibrous	Scaly, rough to the touch, brown on the outter sur- face and white in its inner surface

Table 2. Macroscopy of the powder of *Albizia ferrugiena* pulps.

Pulverised pulps	Color	Strenght of the odor	Odor	Foreign elements
Albizia ferruginea	Brown	Distinct	Aromatic	Absence of foreign elements, high presence of fibers

Table 3. Phytochemical composition of pure extract.

Phytochemical class	Hydro ethanolic extract
Alkaloids	+
Polyphenols	+
Flavonoids	+
anthocyanins	+
Saponins	-
Terpenes	++
Mucilage	+

mL of extract was added to 2 mL of chloroform. By adding 1 mL of acetic anhydride associated with 2 drops of sulfuric acids led to a formation of a ring colored orange.

Interpretation

In reference to the African pharmacopeia, no foreign element was observed (Tables 1 to 3), hence the good quality of the vegetal drugs used. In the microscopic analysis, the powders of Figures 3, 4 and 5 showed the crystal of calcium oxalate and fibers, the pericyclic fibers, the spiral wooden vessels, the clustered sclerotic cells and the suber. Using anoptical microscope, we further confirm the presence of a fibrous structure, previously seen with the naked eyes. It became optimal when it is done using potash as shown by Kaur et al. (2015), who observed sclerotic cells, prisms, the calcium oxalates, oily globules, and fibers.

Those are similar to the work presented by Argarwala et al. (2015) on the powdered pulps which justified the presence of vessels, fibers, grains of starch, xylem and oily globules. The difference observed in the structures could be due to the species.

+ Weakly positive reaction
++ Averagely positive reaction
+++ Highly positive reaction
-Negative reaction

The absence of saponins in the various hydro-alcoholic extracts of pulps can be justified with the type of solvent used. According to Drut-Grevoz (Drut-Grevoz and Laubriet, 2007), using a mixture of ethanol/water (70/30) v/v as a solvent during the extraction lead to a reduction of the polarity of the oxygen by an induction effect. This, therefore, affects the solubility of saponins with the hydro-alcoholic mixture, hence the absence of saponins in the hydro-alcoholic extracts. The loss due to desiccation as defined by World Health Organization (1998), was obtained to be 7.6% for the *A. ferruginea*, this explains the proper conservation of the vegetal drugs. This is in agreement with the results published by Kaur et al. (2015) showed that the loss due to the desiccation of *A. ferruginea* could be estimated to be 7.05%. We obtained a total percentage of ashes of 1.16% for the powder of *A. ferruginea* pulps, which is very different to the 7.59% obtained by Kaur et al. (2015) in the pulps of *Albizia lebbeck*. This show very small quantity of inorganic materials, which could be linked to the species used. From the elements identified in this work, we draw a monograph of the *A. ferruginea* pulps as shown in Figures 4 and 5.

Conclusion

The standardization is an important step when it comes to the evaluation and purity of a given sample. The

macroscopic and microscopic characterization is amongst the most cost efficient used to established and identified vegetal drugs. Whereas, the phytochemical analysis help to confirm those finding. The information and data recorded throughout this study will be of capital importance during the establishment of our national pharmacopeia.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abayomi S (2010). Plantes médicinales et médecine traditionnelle d'Afrique. Editions Karthala 384p ISBN-10: 2811103309 ISBN-13: 978-2811103309.

Abbiw DK (1990). West African uses of the world and cultivated plants, Intermediate Technology Publications, United Kingdom. 203:178.

Abondo-Ngono R, Tchindjang M, Essi MJ, Ngadjui BT, Beyene V (2015). Cartographie des acteurs de la médecinetraditionnelle au Cameroun:cas de la région du centre, Ethnopharmacologia 53:56-63.

Agarwala V, Chauhanb B, Chaurasiab H, Bhardwaj P (2015). Comparative pharmacognostic investi- gation of stems and barks of *Albizia lebbeck*. Int. J. Pharm. Sci. 1(2):46-52.

Alzoreky NS, Nakahara K (2003). Antibacterial activity of extracts of some edible plants commonly consumed in Asia. Int. J. Food Microbiol. 80:223-230.

Ayoola GA, Coker HAB, Adesegun SA, Bello AA, Obaweya K, Ezennia EC (2008). Phytochemical screen- ing and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. Trop. J. Pharm. Res. 7:1019-1024.

Bagre I, Bahi C, Gnahoue G, Djaman AJ, Guede GF (2007). Composition phytochimique et évalua- tion in vitro de l'activité antifongique des extraits des feuilles de morindaMorindoides (baker) milne-redhead (rubiaceae) sur *Aspergillus fumigatus* et *Candida albicans*. Int. J. Sci. Pharm. Biol. Sci. 8(1):15-23.

Blond A, Boutefnouchet S, Cachet X, Cottet K, Genta-Jouve G, Grougnet R (2014). Guide de Travaux Pratiques Pharmacognosie, 3ème année. 2013-2014. Disponible sur www. phar- macognosie - parisdescartes.fr.

Drut-Grevoz G, Laubriet A (2007). Reconnaissance et pr´eparations de m´edicaments `a l'officine Paris, Maloine, 230-231.

Guedouari R (2012). Étude comparative de la pharmacognosie des différentes parties du Laurus- nobilisEssais de formulations thérapeutiques. Mémoire de Magister en Génie des Procédés Chimiques et Pharmaceutiques Option: Industrie Pharmaceutique. Université M'hamedBugara- Boumerdes 2011/ 2012.

Kaur H, Harisha CR, Galib R, Prajapati PK (2015). Pharmacognostical and preliminary physico- chemical profile of Albizzia lebbeck benth tem bark. Global J. Res. Med. Plants Indigenous Med. 4(11):226-235.

Kouadio NJ, Guessennd NK, Kone NW, Moussa B, Koffi YM, Guédé KB (2015). Evaluation de l'activité des feuilles de *Mallotus oppositifolius* (Geisel.) Mül.-Arg (Euphorbiaceae) sur des bactéries multirésistantes et criblage phytochimique. Int. J. Biol. Chem. Sci. 9(3):1252-1262.

M´ethodes de pharmacognosie,Pharmacop´ee europ´eenne 6`eme ´edition (2008). Pharmacopée européenne 6ème édition.

Observatoire de la santé en Afrique (2003). Médecine traditionnelle, Revue du bureau régional de l'OMS pour l'Afrique.

Organisation Mondiale de la Santé (2006). Maladies infectieuses en Afrique. Situation et perspec- tives d'action. 7ème Réunion du forum pour le partenariat avec l'Afrique. Moscou, Russie.19p.

Organisation Mondiale de la Santé (2014). Stratégie de l'OMS pour la médecine traditionnelle pour 2014-2023.72p.

Pélissier Y (2012). *Travaux pratiques de pharmacognosie micrographie*. Faculté des Sciences Pharmaceutiques et Biologiques, Université de Montpellier.

Surendra KR, Shashank B, Suresh D, Aanchal J (2013). Preliminary phytochemical screening of medicinal plant *Ziziphus Mauritiana* Lam. Fruits Int. J. Current Pharm. Res. 4:160-162.

World Health Organization (1998). Quality control methods for medicinal plant materials.

Full Length Research Paper

Diterpenes of the pimarane type isolated from *Viguiera arenaria*: Promising *in vitro* biological potential as therapeutic agents for endodontics

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Viguiera arenaria, **family Asteaceae, is a plant that contains diterpenoids, which make this species potentially applicable in endodontics. More specifically,** *V. arenaria* **contains diterpenes of the pimarane type, which display various classic biological activities. This study evaluates the antibiofilm activity, the time-kill curve, and the inhibitory concentration index of diterpenes of the pimarane type (***ent*-**pimara-8(14),15-dien-19-oic acid,** *ent*-**8(14),15-pimaradien-3β-ol, and** *ent*-**8(14),15-pimaradien-3β-19-oic acid sodium salt, designated diterpenes I, II, and III, respectively) toward nine anaerobic bacteria commonly found in endodontic infections; this study also assesses the cytotoxic activity of these diterpenes against human fibroblasts. According to the antibiofilm assay, diterpenes I**, **II, and III inhibit at least 50% of all the bacteria. On the basis of the time-kill curve experiments, the behavior of these diterpenes depends on the tested bacteria, diterpene concentration, and microorganism sensitivity. Synergism of diterpenes I and II with chlorhexidine (CDH) was higher against** *P. gingivalis* **(clinical isolate) and** *Aggregatibacter actinomycetemcomitans* **(ATCC). As for diterpene III, synergism with CDH is higher against** *P. micros.* **As revealed by the XTT assay, none of the diterpenes of the pimarane type tested here are cytotoxic. Hence, diterpenes I**, **II, and III are promising biomolecules and may provide therapeutic solutions in the field of endodontics.**

Key words: Antibacterial activity, Asteraceae, cytotoxicity, diterpenes, endodontic infection.

INTRODUCTION

For thousands of years, medicinal plants have been used to treat numerous human diseases worldwide. In rural areas, these plants still constitute the primary source of medicine (Chitme et al., 2004; Palombo, 2011). Natural products derived from medicinal plants have been proven as abundant source of active compounds and have been
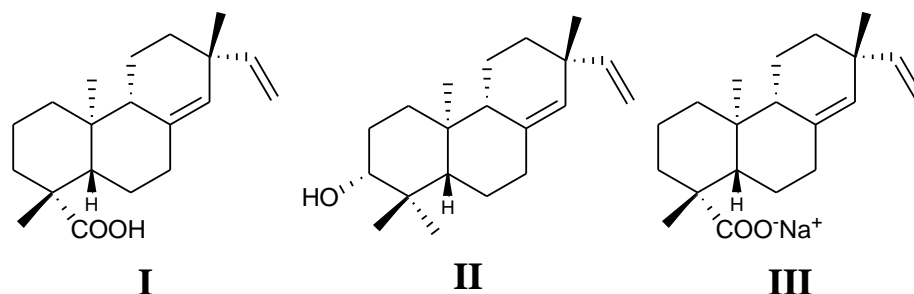
Figure 1. Chemical structures of the diterpenes assayed in this study. **I**, *ent*-pimara-8(14),15-dien-19-oic acid; **II**, *ent*-8(14),15-pimaradien-3β-ol; **III**, *ent*-pimara-8(14),15-dien-19-oic acid sodium salt.

the basis for the development of new chemicals for pharmaceutical products (Palombo, 2011). In this scenario, professionals in the area of dentistry cannot overlook the current perspective of treatment based on compounds originating from novel plant-derived bioactive molecules (Palombo, 2011).

Viguiera arenaria, a herbaceous plant belonging to the family Asteraceae, is native to the Brazilian savannah (Schilling et al., 2000; Ambrósio et al., 2004; Tirapelli et al., 2005; Mizokami et al., 2016). The taxonomic, chemical, and biological features of this plant have been little studied in Brazil. Diterpenes, which are an important class of plant-derived natural products with a wide range of relevant biological activities (Ambrosio et al., 2006; Tirapelli et al., 2008; Veneziani et al., 2017), have been isolated from the *V. arenaria* root extract (Bohlmann et al., 1981; Meragelman et al., 1996; Da Costa et al., 1996; Vaccarini et al., 1999).

Many reports have shown that this class of secondary metabolites exhibits an array of biological actions such as antiparasite effects (Ambrosio et al., 2008; Batista et al., 2007; Da Costa et al., 1996), vascular smooth muscle contraction inhibition (Ambrosio et al., 2006; Ambrosio et al., 2002; Tirapelli et al., 2008), analgesic and anti-inflammatory activities (Okuyama et al., 1991; Paiva et al., 2002; Suh et al., 2004), and relatively selective cytotoxicity toward cancer cells (Ghisalberti, 1997), among other actions. Diterpenes also display significant antimicrobial activity (Tatsimo et al., 2005; Kuzma et al., 2007; Ndi et al., 2007; Fukumoto et al., 2008; Porto et al., 2009a; Porto et al., 2009b; Carvalho et al., 2011; Andrade et al., 2011; Moreira et al., 2016; Moreti et al., 2017; Veneziani et al., 2017).

Ambrosio et al. (2004) isolated and identified diterpenes of the pimarane type from *V. arenaria* baker root extracts. These molecules have potential application in dentistry. According to Carvalho et al. (2011), the antimicrobial properties of these compounds could encourage the development of dental materials for use in endodontics, especially in cases that chronic periapical infections treatment is unsuccessful.

Primary endodontic infection is the infection of the

necrotic root canal and is the prime cause of apical periodontitis (Siqueira et al., 2002; Roças et al., 2011). This infection is chacacterized by anaerobic bacteria that constitute a readily mixed biofilm (Roças et al., 2011; Ricucci and Siqueira, 2010) of polymicrobial origin. Gram-negative bacteria are the main bacteria causing this infection (Fabricius et al., 1982; Caetano da Silva et al., 2014).

Despite the promising results regarding the action of diterpenes of the pimarane type against some potentially pathogenic bacteria in the oral cavity, their antimicrobial effects on bacteria that cause endodontic infections are little known. Biological assays on diterpenes of the pimarane type could provide useful information for the development of new biocampatible materials that can inhibit the growth of bacterial biofilms responsible for endodontic infections.

Notwithstanding the extensive literature on diterpenes, there are not many studies on the activity of these compounds. Therefore, the present study aimed to investigate the antibacterial and antibiofilm activity of diterpenes of the pimarane type against bacterial strains that cause endodontic infections. This study also evaluates diterpene cytotoxicity to ensure that these compounds are safe for subsequent application.

MATERIALS AND METHODS

Obtaining *ent*-pimarane diterpenes from *Viguiera arenaria*

Ent-pimara-8(14),15-dien-19-oic acid (I) and *ent*-8(14),15-pimaradien-3β-ol (II) were isolated from *V. arenaria*. *Ent*-pimara-8(14),15-dien-19-oic acid sodium salt (III) was obtained by semi synthesis from I. All the isolation, identification, and semi synthetic procedures are detailed in Carvalho et al. (2011). Figure 1 shows the chemical structures of compounds I-III. Carvalho et al. (2011) reported the *in vitro* antibacterial activity of diterpenes obtained from *V. arenaria*. Here, we decided to expand the *in vitro* assays to confirm the potential action of said diterpenes.

Assayed bacteria

The bacteria were obtained from the American Type Culture

Collection (ATCC), and the clinical isolates were maintained in the culture collection of the Applied Microbiology Research Laboratory (LaPeMA) of the University of Franca, State of São Paulo, Brazil, under cryopreservation (-80°C) in Brucella broth containing glycerol at 20% (v/v) and 0.5 mL of defibrinated sheep blood. The following bacteria were used: *Porphyromonas gingivalis* (ATCC 33277 and clinical isolate), *Prevotella intermedia* (clinical isolate), *P. nigrescens* (ATCC 33563), *P. buccae* (clinical isolate), *Actinomyces naeslundii* (ATCC 19039), *Bacteroides fragilis* (ATCC 25285), *Peptostreptococcus micros* (clinical isolate), and *Aggregatibacter actinomycetemcomitans* (ATCC 43717).

The clinical isolates were isolated from patients with primary endodontic infections and had been obtained during a previous research work conducted in the dental clinic of the University of Franca. The clinical isolates were isolated, identified, and kept at LaPeMA/UNIFRAN and at the State University of Campinas (UNICAMP), Brazil.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The microdilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2007), was used to determine the MIC. The experiments were conducted in triplicate. The diterpenes were dissolved in dimethyl sulfoxide (DMSO) at 1.0 mg/mL, followed by dilution in Brucella broth (DIFCO, Kansas City, MO, USA) supplemented with hemin (5.0 mg/mL, Sigma, St. Louis, MO, USA) and menadione (1 mg/mL, Sigma) for anaerobic bacteria; and *A. actinomycetemcomitans* were diluted in Brain Heart Infusion broth (BHI; Difco Labs, Detroit, MI, USA). Then, 12 diterpene concentrations ranging from 0.25 to 80 µg/mL were tested.

The inocula were adjusted to give a cell concentration of 5×10^5 CFU/mL for *A. actinomycetemcomitans* (CLSI, 2009) and 1×10^6 CFU/mL for anaerobic bacteria (CLSI, 2007). DMSO 5% (v/v) was used as the negative control, and chlorhexidine dihydrochloride (CDH) was used as the positive control. An inoculum was included to monitor ground for bacterial growth. The 96-well microplate containing *A. actinomycetemcomitans* was incubated in microaerophilic conditions at 37°C for 24 h, and the plates containing anaerobic microorganisms were incubated at 36°C for 72 h in an anaerobic chamber containing 5-10% H_2, 10% CO_2, and 80-85% N_2 (Don Whitley Scientific, Bradford, UK). After incubation, 30 µL of a 0.02% aqueous resazurin (Sigma) solution was added to each well. Resazurin is an oxireduction probe that allows immediate microbial growth observation. The blue and red colors represent microbial growth absence and presence, respectively (Sarker et al., 2007).

To determine the minimum bactericidal concentration (MBC), a 10-µL aliquot of the inoculum was removed from each well before resazurin (Sigma) was added, and the aliquot was seeded in blood agar supplemented with 5% defibrinated sheep blood for microaerophilic strains. For anaerobic bacteria, Schaedler agar (Difco) supplemented with hemin (5 mg/mL, Sigma), menadione (1 mg/mL, Sigma) and 5% defibrinated sheep blood (Bio Boa Vista, Valinhos, SP, Brazil) was used. The agar plates were incubated under gaseous conditions for an appropriate time. After incubation, MBC was defined as the lowest diterpene concentration that killed > 99.9% of the initial bacterial population, where no visible bacterial growth was observed after the subculture in Schadler agar plates. The MBC assays were performed in triplicate.

Biofilm formation inhibition as assessed by minimum inhibitory concentration of biofilm (MICB$_{50}$)

The minimum inhibitory concentration of biofilm (MICB$_{50}$) is defined as the minimum antibacterial agent concentration that is able to inhibit 50% or more of biofilm formation (Wei et al., 2006). MICB$_{50}$ was determined as described in international guidelines (CLSI, 2007) with some modifications. To determine MICB$_{50}$ of the three diterpenes, serial dilutions were accomplished in a polystyrene tissue 96-well plate (TPP, Trasadingen, Switzerland) containing Brucella (Difco) broth supplemented with hemin (5 mg/mL, Sigma, St. Louis, MO, USA) and menadione (1 mg/mL, Sigma) for the anaerobic bacteria (medium 1), and Brain Heart Infusion (BHI, medium 2) broth for *A. actinomycetemcomitans*. The final diterpene concentrations varied from 0.195 to 400 µg/mL. Chlorhexidine dichlorohydrate (CDH, Sigma) was used as positive control, at concentrations ranging from 0.115 to 59 µg/mL. Bacterial strains in the absence of antimicrobials were used as negative controls.

After incubation at 36ºC for 72 h in anaerobic chamber containing 5-10% H_2, 10% CO_2, and 80-85% N_2 (atmosphere 1) for the anaerobic bacteria, and at 37°C for 24 h for *A. actinomycetemcomitans* in microaerophilia (atmosphere 2), the well contents were removed. Then, each well was washed three times with 200 µL of sterile Milli Q water and fixed with 200 µL of methanol for 15 min. MICB$_{50}$ was determined in triplicate.

Following the procedures described by Sandberg et al. (2008), optical density (OD) quantification in the biofilm was conducted by adding 200 µL of crystal violet (0.1%) to the microplate wells. After 10 min at room temperature, excess dye was removed with tap water and dried in air at room temperature. Next, 200 µL of ethanol 95% was slowly added to each well, to re-solubilize the dye bound to the cells. The microtitration plate was covered with a lid and kept at room temperature for at least 30 min, to minimize evaporation. The OD of each well was measured at 595 nm with a microtitration plate reader, and the inhibition percentage was calculated by using the equation (Wei et al., 2006):

$$(1 - At_{595} / Ac_{595}) \times 100$$

Where, At_{595} nm and Ac_{595} nm are the absorbances of the well treated with a diterpene and the control, respectively.

The best incubation time and inoculum concentration for the antibiofilm activity assay were selected by standardizing biofilm formation (data not presented).

Diterpenes of the pimarane type bactericidal kinetics ("time-kill curve")

The time-kill curves were constructed, in triplicate, to determine the time that was necessary for the diterpenes and CDH to inhibit bacterial growth completely (D'Arrigo et al., 2010), as previously described by Moraes et al. (2016). The tubes containing 1 mL of medium 1 for the anaerobic bacteria or 1 mL of medium 2 for the microaerophilic bacterium and one of the diterpenes at their minimum bactericidal concentration (MBC, from 1.0 to 60.0 µg/mL) were inoculated with the assayed microorganisms and incubated in atmosphere 1 or 2 for 72 or 24 h in the case of anaerobic and microaerophilic bacteria, respectively. After incubation, 100-µL aliquots were removed from the tubes at zero, 6, 24, 48, and 72 h for the anaerobic bacteria and at zero, 30 min, and 6, 12, and 24 h for *A. actinomycetemcomitans*.

Seven decimal serial dilutions were then prepared, and 50 µL of each concentration was spread on supplemented Schaedler agar and blood agar for the anaerobic and microaerophilic bacteria, respectively. After the incubation time, the viable colonies were counted, and time-kill curves were constructed by graphical representation of Log_{10} CFU/mL as a function of time, with the aid of the software Prism (versão 5.0; GraphPadV). CDH at concentrations ranging from 0.922 to 7.38 µg/mL was added as positive control.

Table 1. *In vitro* antibacterial activity (MIC and MBC - µg/mL) of diterpenes from *Viguiera arenaria* against endodontic anaerobic bacteria.

Bacteria	Diterpene I		Diterpene II		Diterpene III		CDH	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
P. gingivalis (ATCC 33277)	1.25	10.0	10.0	10.0	1.0	8.0	0.92	0.92
P. gingivalis (clinical isolate)	2.0	10.0	10.0	40.0	1.25	8.0	1.85	1.85
P. nigrescens (ATCC 33563)	2.0	2.0	10.0	40.0	1.5	1.75	0.92	0.92
P. intermedia (clinical isolate)	1.0	1.0	7.5	40.0	1.75	2.0	0.92	0.92
P. buccae (clinical isolate)	4.0	4.0	4.0	14.0	2.25	5.0	0.92	0.92
B. fragilis (ATCC 25285)	2.0	2.0	2.5	2.5	10.0	14.0	7.38	7.38
A. naeslundii (ATCC 19039)	1.25	10.0	5.0	5.0	5.0	5.0	1.85	1.85
P. micros (clinical isolate)	0.5	1.0	6.0	6.0	8.0	12.0	1.85	1.85
A. actinomycetemcomitans (ATCC 43717)	4.0	4.0	10.0	60.0	1.25	1.75	7.38	7.38

Fraction inhibitory concentration index (FICI) determination for CDH and diterpenes of the pimarane type

The checkerboard assay was derived from the standard procedure established by the CLSI (2007) to investigate the antimicrobial efficacy of the *in vitro* association of the diterpenes with CDH that provided the most satisfactory results. The assays were performed according to the protocol previously described by Chaturvedi et al. (2008). The synergy tests were evaluated in triplicate, and the concentrations of diterpenes **I**, **II**, and **III** and CDH were combined in the MIC standard format.

To evaluate the synergism effect (FIC), index values were calculated as previously established in the literature, according to Chaturvedi et al. (2008). The combination was algebraically calculated to determine the fractional inhibitory concentration (FIC) index. FIC_A was calculated as the MIC of drug A in the combination/MIC of drug A alone; FIC_B was determined as the MIC of drug B in the combination/ MIC of drug B alone. The FIC summation (ΣFIC) was calculated as ΣFIC index= $FIC_A + FIC_B$ (Chaturvedi et al., 2008).

The FICI values were analyzed as follows: FICI ≤ 0.5, synergism; FICI > 0.5 and < 1.0, additive; FICI ≥ 1.0 and < 4.0, indifferent; and FICI ≥ 4, antagonism (Lewis et al., 2002).

Cytoxic activity of diterpenes of the pimarane type to human fibroblasts as assessed by the XTT assay

The cell line GM0749-A, corresponding to normal human lung fibroblasts, was employed in this assay. The GM0749-A cells were stored in liquid nitrogen at -195°C. The aliquots contained 1×10^6 cells /mL of a freezing solution consisting of 50% culture medium (HAM F10 + DMEM at 1:1 ratio, Sigma), 40% fetal bovine serum (Nutricell), and 10% DMSO.

For the experiments, the cells were thawed and placed in culture medium (HAM F10 + DMEM at 1:1 ratio) supplemented with 10% fetal bovine serum (Nutricell), 1.2 g/mL sodium bicarbonate, 0.1 g/mL streptomycin (Sigma), and 0.06 g/mL penicillin (Sigma).

Cell monolayers were cultured in 10 mL of culture medium by using disposable flasks with 25-cm² area (Corning) at 37 °C in a BOD incubator. Every 2-3 days, the cells were sub-cultured by using PBS (for washing) and ATV (Instituto Adolfo Lutz, at ATV/PBS 1:1 ratio) to release the cells adhered to the culture flask internal surface. After the cells were released, about 1.5 mL of complete culture medium was added to the flask to inactivate ATV, which was followed by homogenization. A small amount of the cells was then placed in culture flasks containing 5 mL of culture medium and incubated at 37°C.

The cells were sub-cultivated, and approximately 10^4 cells were seeded in microplates containing 100 µL of culture medium (HAM F10 + DMEM at 1:1 ratio). The cells were treated with diterpene I or II at concentrations varying from 0.3125 to 640 µg/mL, or with diterpene III at concentrations ranging from 0.218 to 448 µg/mL. They were then dissolved in DMSO (0.25%) and incubated at 37ºC for 24 h. The negative control (culture medium only), solvent control (DMSO 0.25%), and positive control (DMSO 25%) were included. After treatment, the cells were washed with PBS, which was followed by addition of 100 µL of medium culture HAM F10 without phenol red and 25 µL of XTT (sodium 3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate). After incubation at 37ºC for 17 h, the absorbances were read at 450 and 620 nm on a microplate reader. The cytotoxic activity was evaluated by using the inhibition parameter of 50% cell line growth (IC_{50}). To compare the different treatments, statitstical analyses of the results were accomplished by analysis of variance (ANOVA) followed by the Tukey test.

RESULTS

Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) determination

The MIC and MBC values obtained for diterpenes I, II, and III against the tested bacteria varied from 0.5 to 10, 2.5 to 60, and 1.0 to 14 µg/mL, respectively (Table 1).

Diterpene I exhibited MIC and MBC values of 1.0 µg/mL against *P. intermedia*, 2.0 µg/ mL against *P. nigrescens* and *B. fragilis,* and 4.0 µg/ mL against *P. buccae* and *A. actinomycetemcomitans*, which represented a bactericidal effect. Diterpene II demonstrated a bactericidal effect against *P. gingivalis* (ATCC), *B. fragilis*, *A. naeslundii*, and *P. micros* with MIC/MBC values of 10.0, 2.5, 5.0, and 6.0 µg/mL, respectively. Diterpene III only exerted a bactericidal effect against *A. naeslundii*, with MIC/MBC values of 5.0 µg/mL (Table 1).

Diterpenes I, II, and III exerted a bacteriostatic effect against the other bacteria. The control drug chlorhexidine,afforded MIC values ranging from 0.92 to 7.38 µg/mL (Table 1).

Table 2. Minimum inhibitory concentration of biofilm (MICB$_{50}$) of the diterpenes against the assayed bacteria.

Bacteria (ATCC/clinical isolate)	Minimum inhibitory concentration of biofilm (µg/mL)			
	Diterpene I	Diterpene II	Diterpene III	CDH
P. gingivalis (ATCC 33277)	25.0	6.25	12.5	3.688
P. gingivalis (clinical isolate)	12.5	12.5	12.5	3.688
P. nigrescens (ATCC 33563)	6.5	6.25	12.5	1.844
P. intermedia (clinical isolate)	6.5	25.0	12.5	3.688
P. buccae (clinical isolate)	12.5	6.25	25.0	1.844
B. fragilis (ATCC 25285)	12.5	12.5	6.25	14.75
A. naeslundii (ATCC 19039)	12.5	6.25	6.25	3.688
P. micros (clinical isolate)	6.25	12.5	6.25	3.688
A. actinomycetemcomitans (ATCC 43717)	6.25	6.25	12.5	14.75

Biofilm formation inhibition as assessed by minimum inhibitory concentration of biofilm (MICB$_{50}$)

Table 2 lists the results obtained during the antibiofilm assays. The minimum inhibitory concentration of biofilm (MICB$_{50}$) varied from 6.25 to 25 µg/mL for diterpenes I, II, and III and from 1.844 to 14.75 µg/mL for CDH. All the evaluated diterpenes inhibited biofilm formation by 50% or more.

As for the antibiofilm activity of diterpene I, MICB$_{50}$ was 6.25 µg/mL against *P. nigrescens*, *P. intermedia*, *P. micros*, and *A. actinomycetemcomitans*; 12.5 µg/mL against *P. gingivalis* (clinical isolate), *P. buccae* (clinical isolate)*, B. fragilis* (ATCC) and *A. naeslundii* (ATCC); and 25 µg/mL against *P. gingivalis* (ATCC) (Table 2).

Diterpene II afforded MICB$_{50}$ of 6.25 µg/mL against *P. gingivalis* (ATCC), *P. nigrescens, P. buccae, A. naeslundii*, and *A. actinomycetemcomitan*; 12.5 µg/mL against *P. gingivalis* (clinical isolate), *B. fragilis*, and *P. micros*; and 25 µg/mL against *P. intermedia* (Table 2). Diterpene III provided CIMB$_{50}$ of 6.25 µg/mL against *B. fragilis, A. naeslundii*, and *P. micros*; 12.5 µg/mL against *P. gingivalis* (ATCC and clinical isolate), *P. nigrescens, P. intermedia*, and *A. actinomycetemcomitans*; and 25 µg/mL against *P. buccae* (Table 2).

Diterpenes of the pimarane type bactericidal kinetics ("time-kill curve")

Figure 2A to I illustrates the bactericidal kinetics for the evaluated bacteria considering colony forming units (CFU/mL) as a function of time.

We considered that the antibacterial action corresponded to a decrease of over 3 Log$_{10}$ in the number of microorganisms. Diterpenes III, III, and I provided a decrease of over 3 Log$_{10}$ in the case of *P. gingivalis* (clinical isolate; Figure 2B), *P. nigrescens* (Figure 2C), and *A. naeslundii* (Figure 2G), respectively, after 6 h of incubation. Diterpene II led to a decrease of over 3 Log$_{10}$ for *A. naeslundii* (Figure 2G) and *P. micros* (Figure 2H) after 24 and 48 h of incubation, respectively.

Diterpenes I and III gave a decrease of over 3 Log$_{10}$ in the case of *P. nigrescens* (Figure 2C) and *P. micros* (Figure 2H) after 24 and 48 h of incubation, respectively.

Fraction inhibitory concentration index (FICI) determination for CDH and diterpenes of the pimarane type

Regarding the combination of CDH with diterpenes I, II, or III against *P. gingivalis* (clinical isolate), the effects were synergic, synergic, and additive, respectively (Figure 3B).

As for the combination of CDH with diterpenes II and III against *A. naeslundii* (Figure 3G), the effects were additive in both cases. CDH combined with diterpene II or III against *P. micros* (Figure 3H) elicited additive and synergic effects, respectively.

Finally, the effect synergic was obtained for *A. actinomycetemcomitans* (Figure 3I). For the other bacteria, combination of CDH with diterpenes I, II or III promoted indifferent or antagonistic actions.

Cytoxic activity of diterpenes of the pimarane type to human fibroblasts as assessed by the XTT assay

We evaluated the antiproliferative effects of diterpenes I, II, and III, at different concentrations, by using the GM0749-A cell line and the XTT assay. None of the diterpenes were cytotoxic at the assayed concentrations (Figure 3A to I).

Only at higher concentrations (Table 3, IC$_{50}$ values of 321.60, 202.30, and 61.26 µg/mL for diterpenes I, II, and III, respectively) were the diterpenes cytotoxic, especially diterpene III, which reduced GM0749-A cell growth during the XTT assay.

DISCUSSION

The results reported by Carvalho et al. (2011) motivated our investigation into diterpenes of the pimarane type:

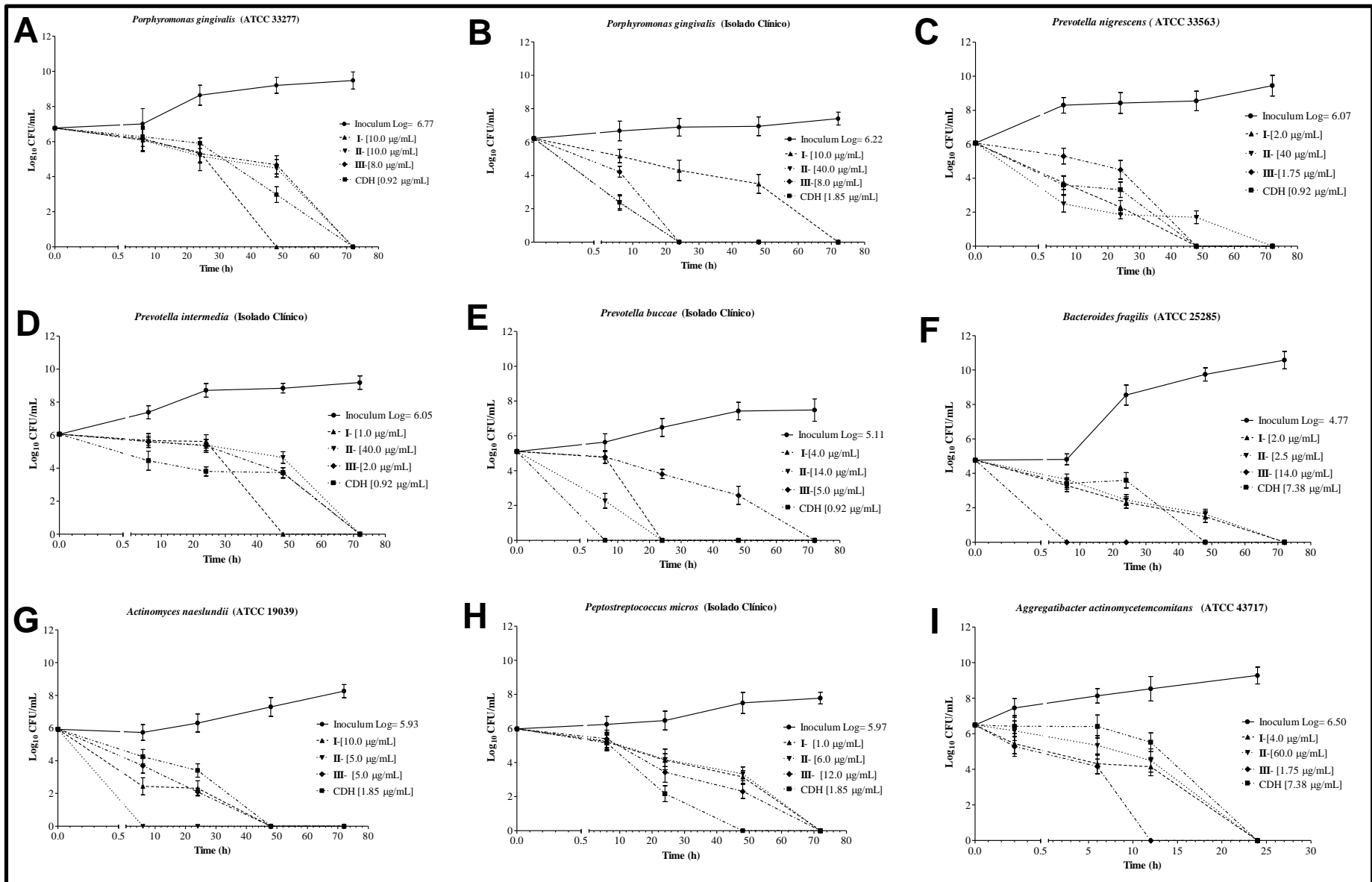**Figure 2.** Time kill- curves against diterpenes **I**, **II** and **III**. Positive control: Chlorhexidine (CDH).
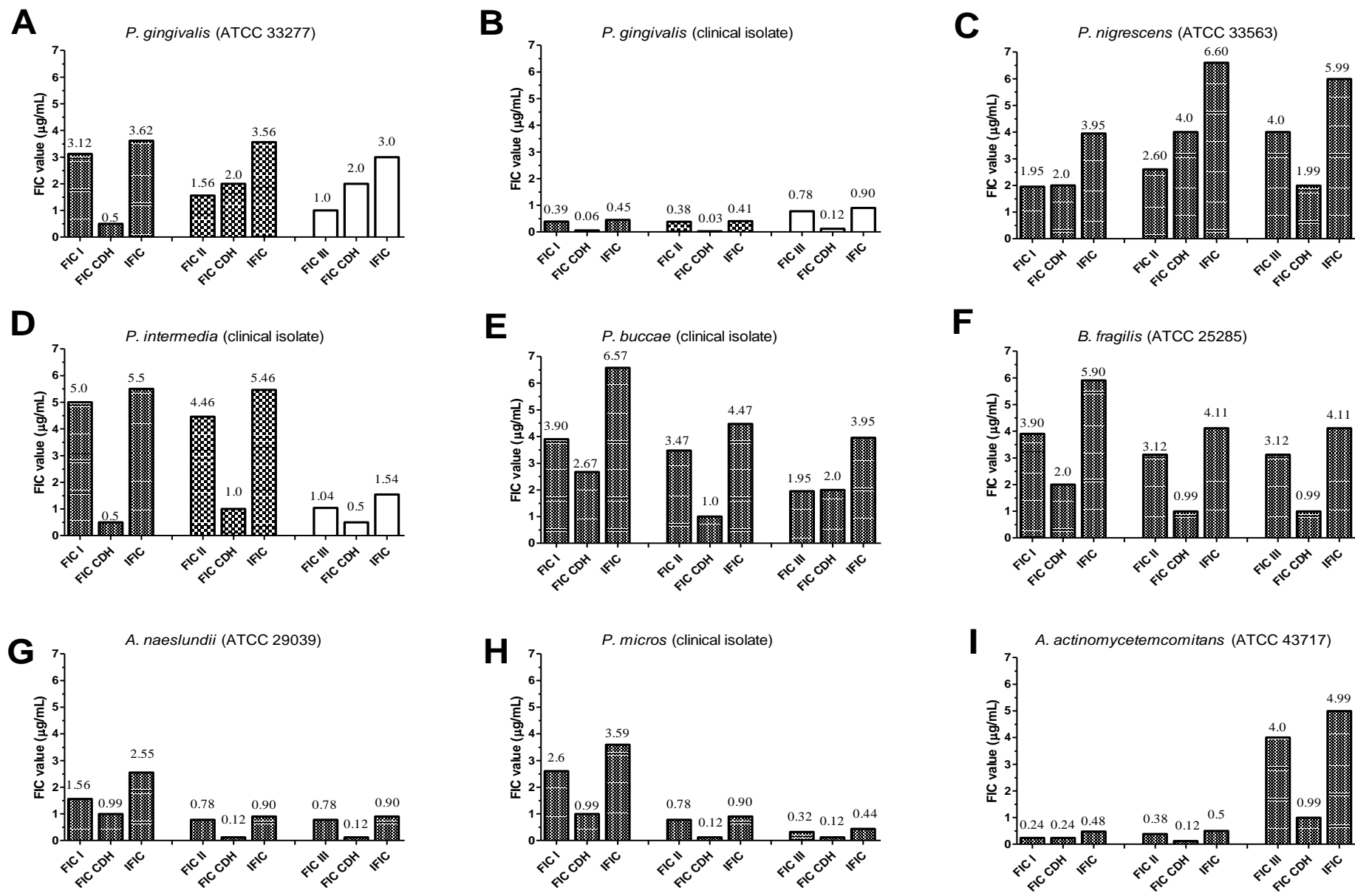
Figure 3. Results for the combination of diterpenes I, II, or III with CDH by the fraction inhibitory concentration index (FICI) method.

Table 3. IC$_{50}$ values obtained for the GM0749-A cell line after 24 h of incubation with different concentrations of diterpenes **I**, **II**, and **III.**

Diterpene	IC$_{50}$ (µg/mL)
I	321.60
II	202.30
III	61.26

these authors found that eight diterpenes had minimum inhibitory concentration (MIC) lower than 10 µg/mL against bacteria that cause endodontic infections. According to the criteria of Rios and Recio (2005), compounds with such MIC values should be promising antibacterials. The plant kingdom is an important source of new and effective antimicrobial agents because plants have the ability to produce natural products for chemical defense against microorganisms present in their own environment (Gibbons, 2004; Hemaiswarya et al., 2008; Gibbons, 2008).

Ambrosio et al. (2006) stated that medicinal plant derivatives like diterpenes of the pimarane type could serve as lead compounds, drugs, or linking structures during the development of synthetic molecules. Furthermore, according to Bakri and Douglas (2005), a new drug can only be considered a therapeutic agent for oral infections if it is active against biofilms.

As part of this scenario, the present paper points out the significant antimicrobial activity displayed by diterpenes isolated from *V. arenaria* against bacteria that cause endodontic infection.

Moreti et al. (2017) evaluated *ent*-kaurenoic acid against endodontic pathogens, to find MIC and MBC values ranging from 3.12 to 400 µg/mL. Souza et al. (2011) investigated copalic acid, a labdane-type diterpene isolated from the *Copaifera langsdorffii* oleoresin, against anaerobic bacteria, to obtain MIC values spanning from 3.1 to 200 µg/mL. In our study, diterpenes I, II, and III afforded MIC values between 0.5 and 10 µg/mL, which agreed with the claim by Rios and Recio (2005) that compounds that can inhibit microorganisms at concentrations close to 10 µg/mL are promising antimicrobials.

Literature reports have described that biofilms can be ten times more resistant to antimicrobials than planktonic cells (Gursoy et al., 2009). According to Signoretto et al. (2011), bacteria in the planktonic and biofilm modes have remarkably different behavior, a fact that must be born in mind during laboratory tests. Moreover, depending on the growth phase, bacterial biofilms respond distinctly to antimicrobial concentration and exposure time (Gursoy et al., 2009; La et al., 2010; Nwaokorie et al., 2010).

The larger bacterial resistance to the diterpenes evaluated herein (MICB$_{50}$ values were higher than MIC values) may have been due to the presence of the extracellular polysaccharide (EPS) matrix, which serves

as a barrier to drug penetration (Molven et al., 1991; Lomçali et al., 1996; Stewart and Costerton, 2001; Ricucci and Siqueira, 2010). Another factor is that biofilm formation inhibition may also inhibit bacterial growth when biofilm formation and bacterial growth are evaluated simultaneously (Wei et al., 2006), as in the case of this work. According to Khemaleelakul et al. (2006), microorganisms residing in biofilms are protected against the toxic effect of antimicrobials via inactivation by enzymes and other metabolites that degrade or neutralize the antimicrobial agent. Here, diterpenes I, II, and III inhibited biofilm formation by the studied endodontic bacteria *in vitro*. Therefore, Wei et al. (2006) believe that avoiding biofilm formation might be more important than destroying the biofilm because the former action should prevent biofilm maturation.

Moreti et al. (2017) evaluated the biofilm activity of the major compound ent-kaurenoic acid, a diterpene isolated from *Mikania glomerata* extract, against *A. actinomycetemcomitans* (ATCC 43717), *P. nigrescens* (ATCC 33563), and *P. gingivalis* (clinical isolate), to obtain 3.12, 6.25, and 200 µg/mL, respectively.

In the present study, the sodium salt of pimaradienoic acid (diterpene III) displayed antibiofilm activity against all the tested microorganisms: MICB$_{50}$ was 12.5 µg/mL against most of the assayed anaerobic species. This bacterial sensitivity to diterpene III is relevant because microbial arrangement in the biofilm mode provides higher bacterial resistance to the root canal chemical-mechanical preparation, especially because the biofilm is located in inaccessible areas of the necrotic pulp in root surfaces and cement gaps (Lomçali et al., 1996; Stewart and Costerton, 2001; Siqueira Jr et al., 2010).

Souza et al. (2011) examined the bactericidal kinetics of copalic acid, a diterpene of the labdane type isolated from the oleoresin of *Copaifera langsdorffii*, against *P. gingivalis* (ATCC 33277). Copalic acid at 3.1 µg/mL presented bactericidal action within 24 h of incubation.

Moreti et al. (2017) also evaluated the time kill curve assay, to detect a bactericidal bactericidal action within 24 h of incubation for *A. actinomycetemcomitans*, 6 h of incubation for *P. nigrescens* and 72 h of incubation for *P. gingivalis* (clinical isolate).

Bactericidal effect is evident when the number of microorganisms decreases by 3 Log$_{10}$ CFU/mL or 99% cell death relative to the initial inoculum occurs within a certain period (May et al., 2000; Shelburne et al., 2004). In the present study, the number of microorganisms diminished by 2 to 3 Log$_{10}$ for some of the assessed bacteria, attesting to the bactericidal effect of the tested diterpenes at the assayed concentrations and time.

Based on the criteria of Lewis et al. (2002), here, the combination of a diterpene with CDH revealed distinct results regarding the interaction between CDH and the tested diterpenes against each of the bacteria. Investigation into standard strains and clinical isolates of the same bacterial species was valid because the biological assays evidenced different behavior between

the strains, as in the case of *P. gingivalis* (ATCC 33277 and clinical isolate). Indeed, combination of CDH with one of the diterpenes was indifferent against the *P. gingivalis* standard strain (ATCC 33277) but synergistic (diterpenes I or III) or additive (diterpene II) against theclinical isolate. Irrespective of the associations tested herein, antagonism was more frequent than synergism for all the diterpenes, indicating that their combination with CDH could in fact limit their action and culminate in undesirable effects.

The results provided by the cytotoxic activity investigation might be as clinically relevant as previous findings for other dose- and time-dependent compounds like CDH (Lessa et al., 2010) and sodium hypochlorite (Pashely et al., 1985; Sabala and Powell, 1989). The *in vitro* cytotoxicity of diterpenes I, II, and III against human fibroblasts might not be the same as the cytotoxicity of these compounds in dental materials or in other experimental conditions, which would require additional *in vitro* and *in vivo* assays. The cytotoxicity of plants, extracts, fractions, and isolated compounds has often been reported to depend on concentration and contact time (Gursoy et al., 2009; Vargas et al., 2010). This toxicity is many times moderate (More et al., 2008; Signoretto et al., 2011) or even uncapable of causing deleterious effects (Seneviratne et al., 2011), but further drug safety assessment is essential before diterpenes of the pimarane type are introduced into the market for use in humans.

Conclusion

Our MIC and MBC results confirmed the data reported by Carvalho et al. (2011). On the basis of the antibiofilm action of diterpenes I, II, and III, these compounds can inhibit at least 50% of the tested bacteria.

The bactericidal kinetic assay evidenced that the behavior of diterpenes I, II, and III against the assayed bacteria varied as a function of diterpene concentration and microorganism sensitivity. Antagonism was the most frequent interaction between CDH and diterpenes of the pimarane type, followed by indifferent, synergistic, and additive interactions.

The cytotoxicity assays demonstrated that diterpenes I, II, and III at different concentrations were not cytotoxic. Diterpene III displayed the most pronounced antiproliferative effect, but this effect emerged at higher concentrations than the ones used during evaluation of the antimicrobial activity of this same diterpene. In conclusion, diterpenes of the pimarane type isolated from plants native to the Brazilian savannah are promising biomolecules and may provide innovative therapeutic solutions in the field of endodontics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ambrosio SR, Arakawa NS, Esperandim VR, Albuquerque S, Da Costa FB (2008). Trypanocidal activity of pimarane diterpenes from *Viguiera arenaria* (Asteraceae). Phytother. Res. 22:1413-1415.

Ambrosio SR, Schorr K, Da Costa FB (2004). Terpenoids of *Viguiera arenaria* (Asteraceae) Biochemical Systematics and Ecology. PloS One 32:221-224.

Ambrosio SR, Tirapelli CR, Bonaventura D, De Oliveira AM, Da Costa FB (2002). Pimarane diterpene from *Viguiera arenaria* (Asteraceae) inhibit rat carotid contraction. Fitoterapia 73:484-489.

Ambrosio SR, Tirapelli CR, Da Costa FB, De Oliveira A (2006). Kaurane and pimarane-type diterpenes from the *Viguiera* species inhibit vascular smooth muscle contractility. Life Sci. 79:925-933.

Andrade BB, Moreira MR, Ambrosio SR, Furtado NA, Cunha WR, Heleno VC, Silva AN, Simão MR, da Rocha EM, Martins CH, Veneziani RC (2011). Evaluation of *ent*-kaurenoic acid derivatives for their anticariogenic activity, Nat. Prod. Commun. 6:777-780.

Bakri IM, Douglas CWI (2005). Inhibitory effect of garlic extract on oral bacteria. Arch. Oral Biol. 50:645-651.

Batista R, Humberto JL, Chiari E, Oliveira AB (2007). Synthesis and trypanocidal activity of *ent*-kaurane glycosides. Bioorg. Med. Chem. 15:381-391.

Bohlmann F, Jakupovic J, Ahmed M, Grenz M, Suding H, Robinson H, King RM (1981). Germacranolides and diterpenes from *Viguiera* species. Phytochemistry 20:113-116.

Caetano da Silva SD, Souza MGM, Cardoso MJO, Moraes TS, Ambrósio SR, Veneziani RCS, Martins RCS (2014). Antibacterial activity of *Pinus elliottii* against anaerobic bactéria present in primary endodontic infections, Anaerobe 30:146-152.

Carvalho TC, Simão MR, Ambrosio SR, Furtado NAJC, Veneziani RCS, Heleno VCG, Da Costa FB, Gomes BP, Souza MG, Borges RE, Martins CHG (2011). Antimicrobial activity of diterpenes from *Viguiera arenaria* against endodontic bacteria. Molecules 16:543-551.

Chaturvedi V, Ramani R, Ghannoum MA, Killian SB, Holliday N, Knapp C, Ostrosky-Zeichner L, Messer SA, Pfaller MA, Iqbal NJ, Arthington-Skaggs BA, Vazquez JA, Sein T, Rex JH, Walsh TJ (2008). Multilaboratory testing of antifungal combinations against a quality control isolate of *Candida krusei*. Antimicrob. Agents Chemother. 52:1500-1502.

Chitme HR, Chandra R, Kaushik S (2004). Studies on antidiarrheal activity of calotropis gigantea R. Br. in experimental animals. J. Pharm. Pharm. Sci. 7:70-75.

Clinical and Laboratory Standards Institute (2007). Methods for antimicrobial susceptibility testing of anaerobic bacteria. CLSI document M11-A7. CLSI, Wayne, Pensilvânia, USA. 7:17-22.

Clinical and Laboratory Standards Institute (2009). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. CLSI document M7-A8. CLSI, Wayne, Pensilvânia, USA. 8:15-18.

D'Arrigo M, Ginestra G, Mandalari G, Furneri PM, Bisignano G (2010). Synergism and postantibiotic effect of tobramycin and Melaleuca alternifolia (tea tree) oil against *Staphylococcus aureus* and *Escherichia coli*. Phytomedicine 17:317-322.

Da Costa FB, Vichnewski W, Herz W (1996). Constituents of *Viguiera aspillioides* and *Viguiera robusta*. Biochem. Syst. Ecol. 24:585-587.

Fabricius L, Dahlén G, Holm SE, Moller A.J (1982). Influence of combinations of oral bacteria on periapical tissues of monkeys, Scand. J. Dent. Res. 90:200-206.

Fukumoto A, Kim YP, Hanaki H, Shiomi K, Tomoda H, Omura S (2008). Cyslabdan, a new potentiator of imipenem activity against methicillin-

resistant *Staphylococcus aureus*, produced by *Streptomyces* sp. K04-0144. II. Biological activities. J. Antibiot. 61:7-10.

Ghisalberti EL (1997). The biological activity of naturally occurring kaurane diterpenes. Fitoterapia 68:303-325.

Gibbons S (2004). Anti-staphylococcal plant natural products. Nat. Prod. Rep. 21:263-277.

Gibbons S., (2008). Phytochemicals for bacterial resistance--strengths, weaknesses and opportunities. Planta Med. 74:594-602.

Gursoy UK, Gursoy M, Gursoy OV, Cakmakci L, Könönen E, Uitto VJ (2009). Anti-biofilm properties of *Satureja hortensis* L. essential oil against periodontal pathogens. Anaerobe 15:164-167.

Hemaiswarya S, Kruthiventi AK, Doble M (2008). Synergism between natural products and antibiotics against infectious diseases. Phytomedicine. 15:639-652.

Khemaleelakul S, Baumgartner JC, Pruksakom S (2006). Autoaggregation and coaggregation of bacteria associated with acute endodontic infections. JOE 32:312-318.

Kuzma L, Rozalski M, Walencka E, Rozalska B, Wysokinska H (2007). Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: salvipisone as a potential antibiofilm agent active against antibiotic resistant Staphylococci. Phytomedicine 14:32-35.

La VD, Howell AB, Grenier D (2010). Anti-*Porphyromonas gingivalis* and anti-inflammatory activities of a-type Cranberry Proanthocyanidins. Antimicrob. Agents Chemother. 54:1778-1784.

Lessa FCR, Aranha FMA, Nogueira I, Giro EMA, Hebling J, Costa CAS (2010). Toxicity of chlorhexidine on odontoblast-like cells. J. Appl. Oral Sci. 18:50-58.

Lewis RE, Diekema DJ, Messer SA, Pfaller MA, Klepser ME (2002). Comparison of test, chequerboard dilution and time-kill studies for the detection of synergy or antagonism between antifungal agents tested against Candida species. J. Antimicrob. Chemother. 49:345-351.

Lomçali G, Sen BH, Cankaya H (1996). Scanning electron microscopic observations of apical root surfaces of teeth with apical periodontitis. Endod. Dent. Traumatol. 12:70-76.

May J, Chan CH, French GC (2000). Time-kill studies of tea tree oils on clinical isolates. J. Antimicrob. Chemother. 45:639-643.

Meragelman KM, Espinar LA, Sosa VE, Uriburu ML, De La Fuente Jr (1996). Terpenoid constituents of *Viguiera tucumanensis*. Phytochemistry 41:499-502.

Mizokami SS, Hohmann MSN, Staurengo-Ferrari L, Carvalho TT, Zarpelon AC, Possebon MI, de Souza AR, Veneziani RC, Arakawa NS, Casagrande R, Verri WA Jr (2016). Pimaradienoic Acid Inhibits Carrageenan-Induced Inflammatory Leukocyte Recruitment and Edema in Mice: Inhibition of Oxidative Stress, Nitric Oxide and Cytokine Production. PLoS One 11:e0149656.

Molven O, Olsen I, Kerekes K (1991). Scanning electron microscopy of bacteria in the apical part of root canals in permanent teeth with periapical lesions. Endod. Dent. Traumatol. 7:226-229.

Moraes TS, Leandro LF, de Oliveira SL, Santiago MB, Souza AB, Furtado RA, Tavares DC, Veneziani RCS, Ambrósio SR, Bastos JK, Martins CHG (2016). *In vitro* evaluation of *Copaifera oblongifolia* oleoresin against bacteria causing oral infections and assessment of its cytotoxic potential. Curr. Pharm. Biotechnol. 17:894-904.

More G, Tshikalange TE, Lall N, Botha F, Meyer JJM (2008). Antimicrobial activity of medicinal plants against oral microorganisms. J. Ethnopharmacol. 119:473-477.

Moreira MR, Souza AB, Soares S, Bianchi TC, de Souza Eugênio D, Lemes DC, Martins CH, Moraes TS, Tavares DC, Ferreira NH, Ambrosio SR, Veneziani RC (2016). *ent*-Kaurenoic acid-rich extract from *Mikania glomerata*: In vitro activity against bacteria responsible for dental caries, Fitoterapia 112:211-216.

Moreti DLC, Leandro LF, Moraes TS, Moreira MR, Veneziani RCS, Ambrosio SR, Gomes BPFA, Martins CHG (2017). *Mikania glomerata* Sprengel extract and its major compound entkaurenoic acid display activity against bacteria present in endodontic infections. Anaerobe 47:201-208.

Ndi CP, Semple SL, Griesser HJ, Pyke SM, Barton MD (2007). Antimicrobial compounds from *Eremophila serrulata*. Phytochemistry 68:2684-2690.

Nwaokorie F, Coker A, Ogunsola F, Gaetti-Jardim Jr E, Gabriel O, Patricia A, Taiwo A, Adesola U (2010). Antimicrobial activities of *Garcinia kola* on oral *Fusobacterium nucleatum* and biofilm. Afr. J. Microbiol. Res. 4:509-514.

Okuyama E, Nishimura S, Yamazaki M (1991). Analgesic principles from Aralia cordata Thunb. Chem. Pharm. Bull. 39:405-407.

Paiva LA, Gurgel LA, Silva RM, Tomé AR, Gramosa NV, Silveira ER, Santos FA, Rao VS (2002). Anti-inflammatory effect of kaurenoic acid, a diterpene from *Copaifera langsdorffii* on acetic acid-induced colitis in rats. Vascul. Pharmacol. 39:303-307.

Palombo EA (2011). Traditional medicinal plant extracts and natural products with activity against oral bacteria: Potential application in the prevention and treatment of oral diseases. J. Evid. Based Complementary Altern. Med. pp.1-15.

Pashely EL, Berdsong NL, Bowman K, Pashley DH (1985). Cytotoxic effects of NaOCl on vital tissue. J. Endod. 11:525-528.

Porto TS, Furtado NAJC, Heleno VCG, Martins CHG, Da Costa FB, Severiano ME, Silva AN, Venezini RC, Ambrósio SR (2009b). Antimicrobial *ent*-pimarane diterpenes from *Viguiera arenaria* against Gram-positive bacteria. Fitoterapia 80:432-436.

Porto TS, Rangel R, Furtado NAJC, De Carvalho TC, Martins CHG, Veneziani RCS, Da Costa FB, Vinholis AH, Cunha WR, Heleno VC, Ambrório SR (2009a). Pimarane-type diterpenes: Antimicrobial activity against oral pathogens. Molecules 14:191-199.

Ricucci D, Siqueira Jr JF (2010). Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. JOE 36:1277-1285.

Rios JL, Recio MC (2005). Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 100:80-84.

Roças IN, José F, Siqueira Jr, Gilbert JD (2011). Analysis of symptomatic and asymptomatic primary root canal infections in adult norwegian patients. JOE 37:1206-1212.

Sabala CL, Powell SE (1989). Sodium hypochlorite injection into periapical tissues. J. Endod. 15:490-492.

Sandberg M, Määttänem A, Peltonen J, Vuorela PM, Fallarero A (2008). Automating a 96-well microtitre plate model *Staphylococcus aureus* biofilms: as approach to screening of natural antimicrobial compounds. Int. J. Antimicrob. Agents 32:233-240.

Sarker SD, Nahar L, Kumarasamy Y (2007). Microtitre platebased antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 42:321-324.

Schilling EE, Da Costa FB, Lopes NP, Heise PJ (2000). Brazilian species of *Viguiera* (Asteraceae) exhibit low levels of its sequence variation. Edinb. J. Bot. 57:323-332.

Seneviratne CJ, Wong RWK, Hagg U, Chen Y, Herath TDJ, Samarayake PL, Kao R (2011). Prunus mume extract exhibits antimicrobial activity against pathogenic oral bacteria. Int. J. Paediatr. Dent. 21:299-305.

Shelburne SA, Musher DM, Hulten K, Ceasar H, Lu MY, Bhaila I, Hamill RJ (2004). *In vitro* killing of community-associated methicillin-resistant *Staphylococcus aureus* with drug combinations. Antimicrob. Agents Chemother. 48:4016-4019.

Signoretto C, Marchi A, Bertoncelli A, Burlacchini G, Tessarolo F, Caola I, Pezzati E, Zaura E, Papetti A, Lingstrom P, Pratten J, Spratt DA, Wilson M, Canepari P (2011). Effects of *Mushroom* and chicory extracts on the physiology and shape of *Prevotella intermedia*, a periodontopathogenic bacterium. J. Biomed. Biotechnol. pp.1-8.

Siqueira Jr JF (2002). Endodontic infections: concepts, paradigms, and perspectives. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 94:281-293.

Siqueira Jr JF, Rôças IN, Lopes HP (2010). Microbiologia endodôntica. In: Lopes HP, Siqueira Jr JF. Endodontia: biologia e técnica. 3th ed. 103-146. Rio de Janeiro: Guanabara Koogan.

Souza AB, Souza MGM, Moreira MA, Moreira MR, Furtado NAJC, Martins CHG, Bastos JK, dos Santos RA, Heleno VC, Ambrósio SR, Venezini RC (2011). Antimicrobial evaluation of diterpenes from *Copaifera langsdorffii* oleoresin against periodontal anaerobic bacteria. Molecules 16:9611-9619.

Stewart OS, Costerton JW (2001). Antibiotic resistance of bacteria in biofilms. Lancelet 358:135-138.

Suh YG, Lee KO, Moon SH, Seo SY, Lee YS, Kim SH, Paek SM, Kim YH, Lee JM, Jeong JM, Lee SJ, Kim SG (2004). Synthesis and anti-inflammatory effects of novel pimarane diterpenoid analogs. Bioorg. Med. Chem. 14:3487-3490.

Tatsimo SJ, Tane P, Sriniva PV, Sondengam BL, Melissa J, Okunji CO, Schuster BM, Iwu MM, Khan IA (2005). Novel antimicrobial diterpenoids from Turraeanthus africanus. Planta Medica 71:1145-1151.

Tirapelli CR, Ambrosio SR, Coutinho ST, de Oliveira DC, da Costa FB, De Oliveira AM (2005). Pharmacological comparison of the vasorelaxant action displayed by kaurenoic acid and pimaradienoic acid. J. Pharm. Pharmacol. 57: 997-1004.

Tirapelli CR, Ambrosio SR, Da Costa FB, De Oliveira AM (2008). Diterpenes: a therapeutic promisse for cardiovascular diseases. J. Pharm. Pharmacol. 3:1-8.

Vaccarini CE, Palácios SM, Meragelman KM, Sosa VE (1999). Phytogrowth-innhibitory activities of a clerodane from Viguiera tucumanensis. Phytochemistry 50:227-230.

Vargas FS, Oliveira CF, Giro EMA, Sacramento LVS, Spolidorio DMP, Costa CAS (2010). Efeito Antimicrobiano e citotóxico do óleo essencial de Cymbopogon citratus sobre células odontoblastóides. Revista Odontológica do Brasil-Central 19:101-107.

Veneziani RCS, Ambrósio SR, Martins CHG, Lemes DC, Oliveira LC (2017). Antibacterial Potential of Diterpenoids. Stud. Nat. Prod. Chem. 54:109-139.

Wei GX, Campgna AN, Bobek LA (2006). Effect of MUC7 peptides on the growth of bacteria and on Streptococcus mutans biofilm. J. Antimicrob. Chemother. 57:1100-1109.

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