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Journal of Medicinal Plants Research

ARTICLES

Full Length Research Paper

Antifungal activities of crude extracts of some Nigerian chewing sticks

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Preliminary screening of both methanol and aqueous extracts of the stem and root of *Distemonanthus benthmianus*, *Treculia africana*, *Garcinia kola* and *Anogessus leiocarpus* was carried out to test for antifungal activity. This was investigated using disc diffusion technique by measuring the zone of inhibition of the fungi after infusion with the plant extract. This produced definite antifungal activity against the dermatophytes used which were *Trichophyton mentagrophytes*, *Candida albicans* and *Aspergillus fumigatus*. *Distemonanthus benthmianus* and *Anogessus. leiocarpus* extracts exhibited antifungal activity at 0.97mg/ml concentration against *Candida albicans* and *Aspergillus fumigatus*. There was significant difference (P<0.05) in fungicidal activity in the methanolic and aqueous extracts. However, between the methanolic and aqueous extracts, there is no significant difference.

Key words: Antifungal, dermatophytes, crude extract, fungicidal activity.

INTRODUCTION

The use of chewing sticks is very popular in Africa, indeed in Nigeria; it is a common practice to see people in local communities using pieces of wood to clean their mouth early in the morning (Adekunle and Odukoya, 2006). This practice has been from time immemorial. It was stated that chewing sticks were once used by the Babylonians and later by the Egyptians, Greeks and Romans. The same report claimed that "toothbrushes" were also common in pre-Islamic Arabia, and that the use of the chewing stick fell out of favor with the advent of Europeans about 300 years ago, yet it is still popular in many parts of Africa, Asia, and the Middle East (William, 2003; Osho and Adelani 2012).

Despite the widespread use of toothbrushes and toothpaste, natural methods of tooth cleaning, using chewing sticks selected and prepared from the twigs, stems, or roots from a variety of plant species have been practiced for many years in Asia, Africa, the Middle East and the Americas (Wu et al., 2001). Darout et al. (2002) reported that selected clinical status have shown that chewing sticks, when properly used, can be as efficient as toothbrushes in removing dental plaque due to the combined effect of mechanical cleaning and enhanced salivation. It has also been suggested that antimicrobial substances that naturally protect plants against various invading microorganisms or other parasites may leach

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out into the oral cavity, and that these compounds may protect the mouth against carcinogenic aniodontopathic bacteria.

Today, chewing sticks are still used in many developing countries because of religion and/or tradition; and because of their availability, low cost and simplicity. The World Health Organization (WHO, 1987) also encourages their use. The year 2000 consensus report on oral hygiene states that chewing sticks may have a role to play in the promotion or oral hygiene, and that evaluation of their effectiveness warrants further research (Axelsson et al., 2002).

In the local communities around the southern part of Nigeria, the use of chewing sticks is very popular, for the cleaning of mouth as part of oral hygiene and also for medicinal purposes especially the use of these plants against oral cavity infections. Some plants used for such purpose include *Distemonanthus benthmianus, Treculia africana, Garcinia kola* and *Anogeissus leiocarpus.*

D. benthomianus is in the family Leguminosae, locally known as "Ayan" (Yoruba) in South Western Nigeria. The twig is used locally or medicinally to stop mouth odour, in cabinetry and decorative veneers (Christine 2003). *T. africana* belongs to the family Moraceae, traditionally called "Ifon" by the Yorubas in Western part of Nigeria where the plant is widely cultivated (Ajayi et al., 2013). *G. kola*, a multi-purpose fruit tree, produces fruit, seeds, roots and stem which are extensively used in Nigeria, Ghana and other West Africa countries for dental care (Emmanuel and Roy, 2001). *G. kola* is a member of the family Sterculiaceae, known locally by Yoruba as "Orogbo" and commonly found in the middle belt areas of Nigeria. *A. leiocarpus*, locally called "Ayin" in Yoruba belongs to the family Combretaceae.

The oral cavity is possible reservoir for microorganisms, both communal and acquired (Hilal and Nizar, 2012). The mouth consists of multiple habitats offering ecological niches to a variety of organisms. Bacteria have been given a notable attention. Fungi have very scanty report despite the documented reports of their association with tooth root infection. Prominent among these reports include Cannon et al. (1995), Waltimo et al. (1997) and Baumgartner et al. (2000). Common trend that runs through the reports is the fact that *Candida albicans* is associated with root canal infection and a constant inhabitant of oral cavity. Another oral infection, thrush is a fungal infection caused by *C. albicans* which forms a white film that irritates the gum and coats the tongue and corners of the mouth and leaves a bleeding surface. Although fungi related infections may not be as common as bacterial infections, they are more difficult to treat especially in patients whose immunity has been compromised. This is one of the reasons the exploration and development of natural products with potent antifungal activity is important (Brooks and Etim, 2004).

The aim of this research was to investigate the antifungal activities of crude extract of *D. benthmianus, G. kola, T. africana,* and *A. leiocarpus* which are commonly used as chewing sticks.

MATERIALS AND METHODS

Source of materials

The stem or root part used as chewing stick were purchased from the local markets and were authenticated at the Department of Pharmacognosy, College of Medicine, Lagos University Teaching Hospital (LUTH) Idi-Araba, Lagos. Information collected from traditional healers and local hawkers from Oyo, Osun, Ogun Ondo and Lagos States of Nigeria included parts of plants used and the preparation and dosage. Local names were also collected. The use of questionnaires and interviews were employed. *Aspergillus fumigatus, C. albicans*, and *Trichophvton mentagrophytes* with accession numbers (014, 003, 016) respectively were all collected from the Department of Medical Microbiology, Lagos University Teaching Hospital (LUTH) Idi-Araba, Lagos. The fungi were stored in Sabouraud Dextrose Agar (SDA) slants at 4°C prior to use, the fungi are clinical samples.

Preparation of discs

Whatman filter paper number one was punched into 200 pieces, 0.6 cm diameter circular discs. This was done using a perforator, the discs were then sterilized. Method of Okigbo et al. (2015) as modified was employed.

Extract preparation

The stem or root of the different plants used as chewing sticks were rinsed under running tap water after which they were dried and chopped into tiny bits. They were then pounded into powdery form with local pistil and mortar. Two hundred (200) grams of the ground plant sample where split into 100 grams for each plant and soaked separately in 70% methanol. For aqueous extract, the plant samples were soaked in water for 5 days (120 h) at room temperature to allow full extraction of all active ingredients (Akande and Ajao, 2011; Osho and Adelani, 2012). The plant materials were placed in a sterilized clinical tube. The fluid were filtered using Whatman filter paper number one (Whatman International Limited, England), after the fifth day.

The extracts were concentrated through evaporation to dryness, under pressure at 50°C using an improvised rotary evaporator (Bankole et al., 2012). The concentrated extract was later kept in the refrigerator for further use.

Culture media

Sabouraud Dextrose Agar (SDA) was used for the culturing of fungi. It was prepared according to the manufacturer's instruction.

Screening for antifungal activity

Liquid inoculum of test fungi was prepared. This was obtained by pouring sterile distilled water into actively growing fungal plates. The fungal inoculum in suspension was carefully dropped onto the already prepared agar plates using sterilized hockey sticks. The pre-sterilized discs were soaked separately in 100 ml of sterile distilled water as control and in both the aqueous and methanol

Table 1. Information obtained from local hawkers

Sample plant	Locality collected	Part of plant	Preparation	Dosage (Tea cup)
Distemonanthus benthmianus	Tejuosho Market	Stem	Wash under running water and chew both the bark and the wood itself	Two times daily (morning and night)
Treculia africana	Ketu Market	Stem	Wash before usage	Two times daily until no more pain
Garcinia kola	Ketu Market	Root	Rinse thoroughly before chewing	Two times daily
Anogessus leiocarpus	Ojo market	Root	Rinse thoroughly before chewing	Two times daily (morning and night)

Table 2. Antifungal activity of methanol extract {zone of inhibition mean ± s.d. (mm)}.

Extract	Organism used	Concentration (mg/ml)						
		0.97	0.49	0.24	0.12	0.06	0.03	0.015
T. Africana	*T. mentagrophyte*	12.00±0.01[a]	10.00±0.60[a]	8.00±0.50[a]	7.00±0.10[a]	5.25±0.05[a]	2.00±0.50[a]	0.01±0.01[a]
	C. albicans	10.00±0.01[b]	9.25±0.20[a]	7.00±0.40[ac]	5.25±0.10[b]	4.00±0.20[b]	2.00±0.20[a]	0.01±0.01[a]
	A. fumigates	10.50±0.50[b]	8.00±0.70[a]	6.25±0.05[bc]	4.00±0.10[c]	3.00±0.10[c]	0.01±0.01[b]	0.01±0.01[a]
G. kola	*T. mentagrophyte*	11.25±0.10[a]	8.00±0.10[a]	6.25±0.05[a]	5.25±0.15[a]	0.02±0.03[a]	0.02±0.01[a]	0.01±0.01[a]
	C. albicans	9.25±0.10[b]	7.00±0.20[b]	6.25±0.10[a]	4.00±0.10[b]	2.00±0.20[b]	0.03±0.02[a]	0.01±0.01[a]
	A. fumigates	9.25±0.10[b]	7.00±0.20[b]	5.25±0.15[b]	5.25±0.15[a]	3.00±0.10[c]	2.00±0.20[a]	0.01±0.01[a]
A. leiocarpus	*T. mentagrophyte*	10.00±0.10[a]	6.25±0.05[a]	5.25±0.05[a]	4.00±0.10[a]	1.00±0.10[a]	0.03±0.01[a]	0.01±0.01[a]
	C. albicans	11.25±0.10[b]	9.25±0.01[b]	6.25±0.05[b]	3.00±0.20[b]	2.00±0.20[b]	0.02±0.01[a]	0.01±0.01[a]
	A. fumigates	10.00±0.10[a]	7.00±0.10[c]	5.25±0.05[a]	4.00±0.10[a]	2.00±0.20[b]	2.00±0.20[b]	0.01±0.01[a]
D. benthmianus	*T. mentagrophyte*	10.00±0.10[a]	8.00±0.10[a]	6.25±0.10[a]	2.00±0.20[a]	2.00±0.20[a]	2.00±0.20[a]	0.01±0.01[a]
	C. albicans	12.30±0.05[b]	10.00±0.10[b]	8.00±0.10[b]	4.00±0.10[b]	4.00±0.10[b]	2.00±0.20[a]	0.01±0.01[a]
	A. fumigates	11.25±0.10[c]	7.00±0.10[c]	5.25±0.05[c]	3.00±0.20[c]	3.00±0.20[c]	2.00±0.20[a]	1.00±0.10[a]
Control	*T. mentagrophyte*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	C. albicans	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	A, fumigatus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Means with the same superscript alphabets and in the same column are not significantly different ($p > 0.05$).

extracts of all the four chewing sticks under study. The discs were soaked for 24 h, after which they were removed using sterile forceps. For each extract, 4 discs were placed separately in the marked Petri-dishes. Seven plates were used for each fungus. Observation was taken at every 24 h to monitor any zone of inhibition on the plates, and measurements were taken as zone of inhibition showed.

Statistical analysis

Data were expressed as mean ± standard deviation. Data were subjected to one-way analysis of variance (ANOVA) (SPSS for Windowns version 17.0) and where there is significant variation, Fisher's Least Significant Difference (LSD) was applied at α = 0.05.

RESULTS AND DISCUSSION

The information collected from traditional healers and local hawkers shows that the parts of the plants used as

chewing sticks includes the stem and the root (Table 1). The fungicidal activity of the plant extracts on the test organisms is shown in (Tables 2 and 3). Both the methanol and aqueous extracts of each of the chewing sticks inhibited the growth of all the fungi. This is in line with previous studies by Bankole et al. (2012) and Osho and Adelani (2012) where both aqueous and ethanolic extracts exhibited growth inhibition against all tested organisms.

Only the extract of *G. kola* had the zone of inhibition less than 10 mm for all the fungi tested at 0.97 mg/ml on both methanol and aqueous extracts and a Minimum Inhibitory Concentration (MIC) of 0.06 mg/ml on methanol and 0.12 mg/ml in aqueous extracts for *G. kola* against *T. mentagrophytes*.

Among the plant extracts, *D. benthmianus* and *A. leiocarpus* had the highest antifungal activity against *C. albicans* on aqueous extract with mean range of 13.0±0.10 mm at 0.97 mg/ml, with a minimum inhibitory

Table 3. Antifungal activity of aqueous extract {zone of inhibition mean ± s. d. (mm)}.

Extract	Organism used	Concentration (mg/ml)						
		0.97	0.49	0.24	0.12	0.06	0.03	0.015
T. Africana	T. mentagrophyte	11.25 ± 0.10^a	9.25 ± 0.20^a	8.00 ± 0.10^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.02 ± 0.01^a	0.01 ± 0.01^a
	C. albicans	10.00 ± 0.10^b	9.25 ± 0.20^a	7.00 ± 0.10^b	5.25 ± 0.15^b	4.00 ± 0.10^b	2.00 ± 0.20^b	0.01 ± 0.01^a
	A. fumigatus	10.00 ± 0.10^b	8.00 ± 0.10^b	6.25 ± 0.05^c	4.00 ± 0.10^c	3.00 ± 0.20^c	0.03 ± 0.02^a	0.01 ± 0.01^a
G. kola	T. mentagrophyte	11.15 ± 0.10^a	8.00 ± 0.10^a	8.00 ± 0.20^a	4.00 ± 0.10^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a
	C. albicans	9.25 ± 0.20^b	7.00 ± 0.10^b	6.25 ± 0.05^b	4.00 ± 0.10^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a
	A, fumigatus	9.25 ± 0.20^b	9.25 ± 0.20^c	8.00 ± 0.20^a	4.00 ± 0.10^a	0.01 ± 0.01^a	0.01 ± 0.01^a	0.01 ± 0.01^a
A. leiocarpus	T. mentagrophyte	10.00 ± 0.10^a	7.00 ± 0.10^a	6.25 ± 0.05^a	3.00 ± 0.20^a	2.00 ± 0.20^a	0.01 ± 0.01^a	0.01 ± 0.01^a
	C. albicans	13.00 ± 0.10^b	10.00 ± 0.10^b	7.00 ± 0.10^b	4.00 ± 0.10^b	2.00 ± 0.20^a	0.01 ± 0.01^a	0.01 ± 0.01^a
	A. fumigatus	11.25 ± 0.10^c	8.00 ± 0.10^c	6.25 ± 0.05^a	4.00 ± 0.10^b	2.00 ± 0.20^a	0.01 ± 0.01^a	0.01 ± 0.01^a
D. benthmianus	T. mentagrophyte	10.00 ± 0.10^a	8.00 ± 0.10^a	6.25 ± 0.05^a	3.00 ± 0.20^a	2.00 ± 0.20^a	2.00 ± 0.20^a	0.01 ± 0.01^a
	C. albicans	13.00 ± 0.10^b	10.00 ± 0.10^b	8.00 ± 0.10^b	6.25 ± 0.05^b	4.00 ± 0.10^b	2.00 ± 0.20^a	0.01 ± 0.01^a
	A. fumigatus	11.25 ± 0.10^c	9.25 ± 0.20^c	7.00 ± 0.10^c	5.25 ± 0.15^c	3.00 ± 0.10^c	2.00 ± 0.20^a	1.00 ± 0.10^b
Control	T. mentagrophyte	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	C. albicans	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	A. fumigatus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Means with the same superscript alphabets and in the same column are not significantly different (p>0.05).

concentration of 0.03 mg/ml in *A. leiocarpus* and no MIC in *D. benthmianus.* In the methanol extract, the highest antifungal activity was observed with *D. benthmianus* and *T. africana* against *C. albicans* and *T. mentagrophytes* respectively, at 0.97 mg/ml for both samples.

For both methanol and aqueous extract, observation shows that the aqueous extract of *D. benthmianus* and *A. leiocarpus* were the most effective of all four chewing sticks investigated for potential treatment of fungal infections in the oral cavity. Ogundiya et al. (2006) also reported that *A. leiocarpus* showed a higher antimicrobial activity

among two other chewing sticks used in that research. The control which was sterile distilled water gave no zone of inhibition against any of the dermatophytes used.

The result presented here showed the presence of antifungal substances in the methanol and aqueous extracts of the chewing sticks used, *D. benthmianus* and *A. leiocarpus* possessed the highest antifungal activity on the test fungi, while *G. kola* showed the least antifungal activity, which properly might be attributed to its low activity. A similar observation was made by Ogundiya et al. (2006) where the extracts of the three chewing

sticks used in the study had no activity on *C. albicans.* Ikenebomeh and Methitiri (1988) also reported that extract of *Casia alata* at various dilutions did not inhibit the growth of *C albicans*, indicating that it might be resistant to some antimycotic agents. However, the result from this study agrees with that of Adejumobi et al. (2008) and Osho and Adelani (2012) that *A. leiocarpus* is active against *C. albicans.*

For both methanolic and aqueous extracts, at concentrations of 0.03 and 0.015 mg/ml, generally there is no significant difference (p>0.05) between the activity of the plant extracts against the test

organisms. However, for *T. africanum* against *A. fumigatus* and *A. leiocarpus* against *A, fumigatus* at 0.03 mg/ml, there is significant difference (p<0.05) in the activity of the methanolic extracts. Also for aqueous extracts, *T. africanum* against *C. albicans* and *D. benthmianus* against *A. fumigatus* at 0.03 and 0.015mg/ml respectively showed significant difference.

Conclusion

The regular use of the African chewing sticks may decrease the incidence of gingivitis and dental carriers by controlling plague formation. Although the report of William (2003) demonstrated that the dental caries is an infection, and so extracts of some sticks have been shown to have antibacterial and antifungal properties to act against these infections in the oral cavity. From this study, it can be deduced that these chewing sticks have the potential of controlling infections caused by these fungi.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antimicrobial effect of chalepensin against *Streptococcus mutans*

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Dental diseases play an important role in public health. The use of conventional antibiotics for treatment can create microbial resistance; therefore, it is critical to search for alternatives to which there is no such an effect. In this regard, we have studied the *in vitro* effect of chalepensin, from the plant *Ruta chalepensis* L., against the dental caries etiological agent *Streptococcus mutans*. *R. chalepensis* is commonly used for treating rheumatism, hypertension, and as a skin antiseptic, anticonvulsant, deworming, and stimulant of menstrual discharge. Antimicrobial effect of chalepensin was measured by the methods of colony forming units (CFU) counts in solid medium culture and reduction of the tetrazolium salt MTT in liquid medium. Chalepensin was shown to cause significant ($p < 0.05$) 53 to 76% and 50 to 71% *S. mutans* growth inhibition at 7.8 to 500 µg/ml in liquid and solid media, respectively, with MICs of less than 7.8 µg/ml. Our results indicated that chalepensin possesses antimicrobial activity against *S. mutans*.

Key words: *Ruta chalepensis*, chalepensin, antibacterial activity, *Streptococcus mutans*.

INTRODUCTION

Dental caries and periodontal disease constitute an important health problem worldwide (Jin et al., 2016; Mattos et al., 1998), involving *Streptococcus mutans* as a major etiological pathogen in dental caries (Becker et al., 2002; Loesche, 1986). In 2013, the World Health Organization reported that billions of people were affected by dental caries; particularly, it is known that

about half of Mexican population has dental diseases that have effects on systemic health (WHO, 2013). Furthermore, periodontal disease is commonly associated with tooth loss (Arweiler and Netuschil, 2016), and it is recognized that microorganisms of the plaque, gingival sulcus microbiota, and their metabolic products are initiators of the disease (Park et al., 2015).
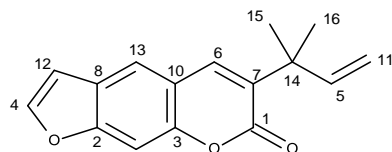
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$C_{16}H_{14}O_3$

Figure 1. Structure of chalepensin.

The urgent need for prevention and control of oral infectious diseases, prompts the development of new alternative treatments, to which resistance has not been developed (Chinedum et al., 2005). The search for plant extracts and active compounds has demonstrated their usefulness as a source of new agents against oral infections (Karygianni et al., 2015); in fact, more than 35% of drugs used in the clinics against infections derive from plants (Choi et al., 2003).

The aim of this study was to evaluate the *in vitro* effect of chalepensin from *Ruta chalepensis* against *S. mutans*.

MATERIALS AND METHODS

Reagents, culture media, and bacteria

Tetracycline solution was purchased from Life Technologies (Grand Island, NY). N-dimethylformamide (DMF), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (St. Louis, MO). Brain heart infusion broth and agar (BHI) were obtained from Difco Laboratories, Inc. (Detroit, MI). *S. mutans* bacteria were acquired from the American Type Culture Collection (Rockville, MD; ATCC700611).

Isolation and identification of chalepensin

R. chalepensis leaves were collected in Escobedo, Nuevo León, México, and identified with the voucher number 025579, at the Herbarium in Facultad de Ciencias Biológicas at Universidad Autónoma de Nuevo León, México. Leaves were dried at 37°C for 5 days, after which, they were ground.

Chalepensin was extracted and identified as previously reported (Quintanilla-Licea et al., 2014). *R. chalepensis* pulverized leaves (600 g) were extracted during 40 h, using methanol in a Soxhlet equipment. Next, the solvent was removed and the extract was dissolved in methanol, after which the solution was partitioned using *n*-hexane. This partition was chromatographed on silica gel and eluted with the consecutive gradients *n*-hexane–chloroform, chloroform–ethyl acetate, and methanol; 8 fractions were obtained, and fraction 2, containing a compound with an R_f of 0.41 was further fractioned using the gradients mentioned above (data not shown; Quintanilla-Licea et al., 2014). This process produced 5 fractions, of which fraction 2 contained a compound with that R_f, which was additionally fractioned with chloroform–ethyl acetate gradients, resulting in 4 main fractions; fraction 2 contained pure chalepensin (Figure 1), which was confirmed by mass spectroscopy and nuclear magnetic resonance (data not shown; Quintanilla-Licea et al., 2014).

Effect of chalepensin on *S. mutans* growth

Fifty microliters of a *S. mutans* suspension (1 x 10³ bacteria/ml) in

BHI broth (Remel, Lenexa, KS) were transferred to flat-bottomed 96-well plates (Corning Incorporated, Corning, NY) containing serial dilutions (1:2) of 50 µl of chalepensin, 1.5 µg/ml tetracycline, and BHI broth controls, and incubated for 6 h at 37°C. Next, MTT was added to all wells (0.5 mg/ml in saline solution, final concentration) and microplate cultures were incubated for 4 additional hours. Next, 50 µl of extraction buffer were added to all wells, microplates incubated (Yamato IC600 incubator) for 16 h at 37°C, and optical densities read at 570 nm (microplate reader, Beckman Coulter, Inc., Fullerton, CA) (Gomez-Flores et al., 1995). Extraction buffer was prepared by dissolving 20% (w:v) SDS at 37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7. For colony forming units (CFU) determination, 1:10,000 dilutions from treatment and control wells, as explained above, were seeded on BHI agar plates (Becton Dickinson, Mexico, D.F.) and incubated for 24 h at 37°C, after which CFU were counted (ULB-100, Scienceware, Pequannock, NJ) (Kansal et al., 1998).

Statistical analysis

The results were expressed as mean ± SE of three replicate determinations from three independent experiments. Statistical significance was assessed by the ANOVA, $p < 0.05$, and pos-hoc Tukey, using SPSS 21.

RESULTS

Characterization of chalepensin

The fractionation of *R. chalepensis* methanolic extract by partition between methanol and n-hexane followed by chromatography of the hexane residue over a silica gel column, produced chalepensin (data not shown; Quintanilla-Licea et al., 2014), whose spectroscopic data were identical with those previously reported by others (Malikov and Saidkhodzhaev, 1998). In addition, the complete assignment of the ¹³C-NMR spectrum of this molecule as the hydrogen and carbon connectivities in 2 were deduced from ¹H-¹H Correlated Spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra, were reported by our research group (data not shown; Quintanilla-Licea et al., 2014).

Inhibition of *Streptococcus mutans* growth by chalepensin

Chalepensin induced significant ($p < 0.05$) 53 to 76% and 50 to 71% growth inhibition of *S. mutans* at 7.8 to 500 µg/ml, as measured by the MTT reduction and CFU methods, respectively, with MICs of less than 7.8 µg/ml (Figure 2). There was not statistical significance between concentrations, as determined by ANOVA, $p < 0.05$, and pos-hoc Tukey. Absorbances at 570 nm (0.58 ± 0.01) resulting from bacterial growth in BHI broth for chalepensin-untreated bacteria, was used as the control value. Tetracyclin control caused 94, 100, 97, 98, 98, 94, 97, and 98% growth inhibition at concentrations of 0.045,
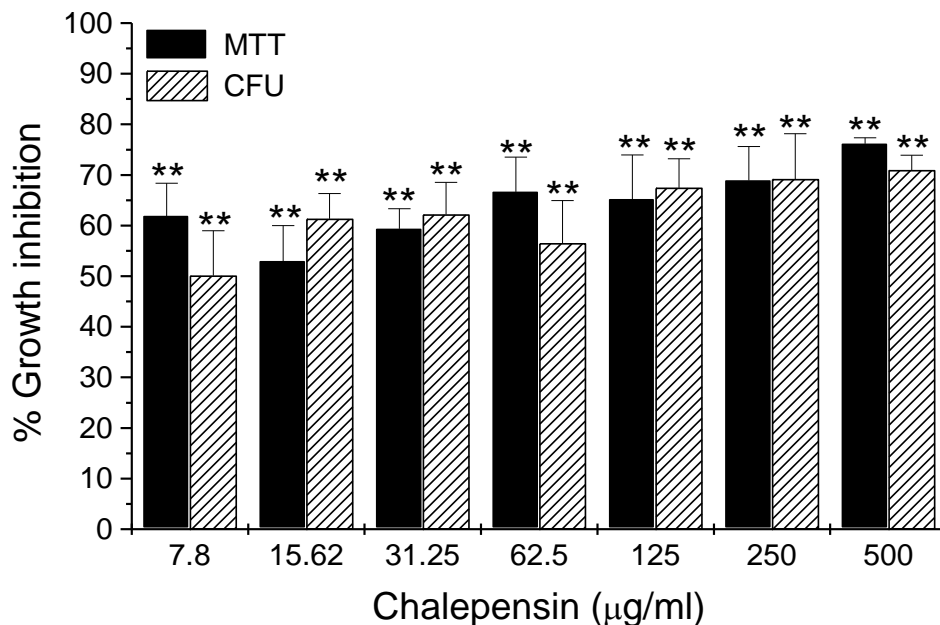
Figure 2. Antimicrobial effect of chalepensin from *R. chalepensis* on *S. mutans* growth. *S. mutans* culture suspensions were incubated with chalepensin, followed by measuring viability by the MTT reduction and CFU methods, as explained in the text. Data indicate the mean ± SE of 3 replicate determinations from 3 independent experiments. **$p < 0.01$, as compared with chalepensin-untreated control. Control absorbance value at 570 nm in liquid medium (BHI broth) for untreated cells was 0.58 ± 0.01, whereas control value for untreated cells in solid medium (BHI agar) was 69.4 ± 5.4 X 10^6 CFU/ml. Tetracyclin control caused 94% bacterial growth inhibition at 1.5 μg/ml.

0.09, 0.185, 0.37, 0.75, 1.5, 3, and 6 µg/ml, respectively (there was not statistical significance between concentrations, as determined by ANOVA, *p* <0.05, and pos-hoc Tukey).

DISCUSSION

Medicinal plants are commonly used, by consumption or directly applied to the injured area, to treat a number of maladies (Rojas et al., 1992). The isolation and evaluation of compounds with antibiotic activity from plants is critical, due to the acquired resistance of pathogens to conventional antibiotics (Chinedum, 2005). Plant-derived anti-infectious agents are usually accepted in a health program if their MICs are 100 to 1000 µg/ml (Drusano, 2004). In this regard, the results of our study, MIC of less than 7.8 µg/ml, demonstrated strong antibiotic activity of chalepensin against *S. mutans.*

Medicinal plants and antimicrobial phytochemicals are known to be useful in controlling dental disease-causing bacteria (Ramakrishnan et al., 2007). Plants such as *Glycyrrhiza glabra, Allium sativum, Aloe vera*, *Physalis angulata, Annona senegalensis, Dryopteris crassirhizoma, Quercus infectoria, Englerophytum magalismontanum, Euclea natalensis, Solanum panduriforme, Rosmarinus officinalis* Linn., *Baeckea frutescens,* and *Parinari curatellifolia* were demonstrated to have antibacterial activity against *S. mutans* (Ban et al., 2012; Fani and Kohanteb, 2012; Hwang et al., 2004; More et al., 2008). In addition, substances such as linoleic, linolenic, oleanolic, betulinic acids, betulin, and beta-sitosterol glucoside, among others, were reported to suppress adherence of *S. mutans in-vitro* (Wu, 2009).

Chalepensin (Figure 1), previously isolated and identified (Quintanilla-Licea et al., 2014), can be found particularly in plants of the Rutaceae family (Günaydin and Savci, 2005), with reported anti-fertility (Kong et al., 1989), antitumor (Wu et al., 2003), and antiplatelet aggregation (Lv et al., 2015) activities. In the present study, we showed for the first time evidence of the *in vitro* antimicrobial effect of chalepensin, isolated from R. *chalepensis*, against *S. mutans*, which broadens its spectrum of biological activity and provides the basis for further validation in pre-clinical studies.

Conclusions

Dental caries is the second most prevalent disease in humans, after the common cold. It is recognized that *S. mutans* overgrowth is the major cause of the disease, for

which, treatments are focused to eliminate this bacterium or controlling its pathogenicity. The basic treatment involves fluoride and antibiotics. However, the use of conventional antibiotics can promote bacterial resistance; it is then essential to investigate for novel antimicrobial agents to which bacteria are not resistant. The increasing research in medicinal plants as a natural source of antibiotics, has produced the discovery of several plant extracts with antimicrobial activity to *S. mutans*. Extracts from *R. chalepensis* were submitted to a fractionation, leading to structure elucidation of isolated compounds by spectroscopy and mass spectrometry. The methanolic extract rendered chalepensin, which caused significant *S. mutans* growth inhibition (up to 76% growth inhibition), with MICs of less than 7.8 µg/ml. To our knowledge, this is the first report on the antibacterial effect of chalepensin against *S. mutans*. This compound may be a potential alternative for treating dental caries or be useful in the development of new antibacterial agents.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Antitumor activity of *Pachycereus marginatus* (DC.) Britton & Rose extracts against murine lymphoma L5178Y-R and skin melanoma B16F10 cells

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Pachycereus marginatus (DC.) Britton & Rose is a species belonging to the family Cactaceae. In traditional medicine, it is recommended to treat diabetes and gastrointestinal infections; however, there are no studies related to its use in cancer treatment. The *in vitro* antitumor effect of *P. marginatus* hexane, chloroform, methanol, and methanol-aqueous partition stem extracts, against murine lymphoma L5178Y-R and skin melanoma B16F10 cells, was evaluated in liquid medium by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The extracts resulted in up to 84, 85, 84, and 82% cytotoxicity ($p < 0.05$) to L5178Y-R cells, respectively, and up to 39, 51, 48, and 42% cytotoxicity ($p < 0.05$) to B16F10 cells, respectively. Vehicle controls were not cytotoxic for tumor cells, and along with the extracts they did not affect viability of resident murine thymus and spleen lymphocytes. Taken together, the present results showed that *P. marginatus* extracts possess antitumor potential against L5178Y-R lymphoma and B16F10 skin melanoma cells.

Key words: Cancer, cacti, *Pachycereus marginatus*, lymphoma, melanoma, medicinal plants.

INTRODUCTION

In the search for alternatives to treat diseases, researchers worldwide have taken the task of finding native plants with potential health benefit. Among them, numerous cacti species have been reported to possess

antitumor properties (Shetty et al., 2012; Harlev et al., 2013; Lema-Rumińska and Kulus, 2014). *Pachycereus marginatus* (DC.) Britton & Rose (Caryophyllales: Cactaceae), also known as *Stenocereus marginatus,Cereus marginata*, Central Mexico organ pipe, organo, Jarritos, Chilayo, and Mexican fencepost cactus, belongs to the Pachycereeae tribe. It has columnar trunks that can reach 20 m high and is commonly used as fodder, living fences, fuel wood, and as an alternative medicine. The species is endemic to Mexico, where it grows wild in states that have a dry and hot climate such as Nuevo León, Guanajuato, Aguascalientes, Oaxaca, Puebla, San Luis Potosí, Zacatecas, Ciudad de México, Tamaulipas, Guerrero, Michoacán, Hidalgo, Jalisco, Morelos, Veracruz, Tlaxcala, and Querétaro (Hernández et al., 2004), and in Texas, New Mexico, Arizona, and Southern California (Paredes-Flores et al., 2007; Arias and Terrazas, 2009). *P. marginatus* is traditionally used to treat gastrointestinal infections (Hernández et al., 2003); its antimicrobial potential in plants, animals, and humans has been reported, as well as its activity to improve wound healing, and promote plant growth (Jordan-Hernandez, 2012). However, the antitumor potential of *P. marginatus* has not yet been reported.

Cancer results from the interaction of internal (as genetic predisposition) and external factors that lead to cell degeneration, resulting in precancerous lesions and ultimately malignant tumors. If it is not promptly treated, cancer cells can spread to other organs (metastasis).

Cancer is one of the main causes of morbidity and mortality worldwide, with about 14 million new cases and 8.2 million deaths in 2012 and new cases are expected to increase 22 million in the next 20 years (Stewart and Wild, 2014). In men, most cancers affect prostate, lungs, and gastrointestinal tissues; in women, cancers in the breast, lung, cervix, and stomach are common (Stewart and Wild, 2014). In Mexico, according to the International Union Against Cancer, cancer is the third leading cause of death and estimated that each year 128,000 new cases are reported (Stewart and Wild, 2014).

The aim of the present study was to evaluate the *in vitro* cytotoxic activity of the hexane, chloroform, methanol, and aqueous methanol partition extracts of *P. marginatus* against murine lymphoma L5178Y-R and skin melanoma B16F10 cells.

MATERIALS AND METHODS

Reagents and culture media

Penicillin-streptomycin solution, L-glutamine, and RPMI 1640 and AIM-V media were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS), concanavalin A (Con A), sodium dodecyl sulfate (SDS), N, N-dimethylformamide (DMF), and 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Vincristine sulfate

was obtained from Laboratorios PiSA (Mexico City, Mexico). The tumor cell lines L5178Y-R (mouse DBA/2 lymphoma R, clone CRL-1722) and B16F10 (mouse skin melanoma) were purchased from The American Type Culture Collection (Rockville, MD), and were maintained in culture flasks with RPMI 1640 medium, supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin streptomycin solution (referred as complete RPMI 1640 medium) at 37°C, in a humidified atmosphere of 5% CO_2 in air; cellular density was kept between 10^5 and 10^6 cells·ml^{-1}. Extraction buffer was prepared by dissolving 20% (w:v) SDS at 37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

Preparation of plant extracts

Pachycereus marginatus used in the present study was identified with voucher number 025588. Stems were rinsed with tap water to eliminate dust and other contaminating material, dried at 37°C for 36 h, and pulverized. Sixty grams of the powdered stems were sequentially extracted with 600 ml of hexane, chloroform, and methanol by Soxhlet system during 40 h each. The extracts were concentrated to dryness using a rotary evaporator Büchi (Brinkmann Instruments Inc., Switzerland) and stored at 6°C until use and dissolved in distilled water for biological activity testing. The methanol-aqueous partition of the extract was prepared by using 11 g of methanol extract and dissolving it in methanol, then in hexane, and shaking for 10 min. The resulting solution was separated with a funnel and washed with ethyl acetate. This solution was washed with water and then dried (referred as aqueous partition). Stock solutions were then prepared at 1 mg ml^{-1} in complete RPMI 1640 (for L5178Y-R cells), AIM-V medium (for lymphocyte cultures) or DMEM medium (for B16F10 cells), and sterilized by filtering through a 0.22-microns membrane (Millipore, Bedford, MA). *P. marginatus* hexane, chloroform, methanol, and methanol-aqueous partition stem extracts were tested at concentrations ranging from 0.03 to 500 µg ml^{-1}.

L5178Y-R and B16F10 cells preparation and culture

In order to determine the direct *in vitro* effect of the extracts on tumor cell growth, L5178Y-R cell and B16F10 cultures were collected and the cellular suspensions obtained were washed three times in RPMI 1640 (for L5178Y-R cells) or DMEM medium (for B16F10 cells), and suspended and adjusted to 5×10^4 cells ml^{-1} in those culture media. One hundred microliters of the cell suspensions were then added to flat-bottomed 96-well plates (Becton Dickinson, Cockeysville, MD), containing 100 µl triplicate cultures of complete RPMI 1640 or DMEM media (unstimulated controls), the extracts at various concentrations (extracts were dissolved in complete RPMI 1640 (for L5178Y-R cells) or DMEM medium (for B16F10 cells), extract-free vehicles (vehicles were similarly processed as with *P. marginatus* extracts, but without plant material), and vincristine as a positive control. After incubation for 44 h at 37°C with 5% CO_2, MTT (0.5 mg ml^{-1}, final concentration) was added, and cultures were additionally incubated for 4 h. Next, cell cultures were incubated for 16 h with extraction buffer (100 µl well^{-1}) and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (DTX 880 Multimode detector, Becton Dickinson, Austria) at 570 nm (Gomez-Flores et al., 2009). The percentage of cytotoxicity was calculated as follows:

% Cytotoxicity = 100-[(A570 in extract treated cells/ A570 in untreated cells) × (100)]

Table 1. Effect of *P. marginatus* extracts on L5178Y-R cells toxicity [%].

Concentration [µg·ml⁻¹]	Hexane	Chloroform	Methanol	Methanol-aqueous partition
0.03	1	0	14.2±1.9	0
0.06	0	0	16.4±1.4	0
0.121	0	0	8.0±0.6	0
0.243	0	0	15.3±2.0	0
0.487	0	0	17.3±2.4	3.0±0.3
0.975	7.8±0.6	0	22.2±4.7	18.1±1.7
1.95	20.0±1.8*	31.7±1.5*	31.9±6.3*	22.1±3.4*
3.9	19.5±2.9*	81.3±6.5**	36.9±6.8*	65.3±9.4**
7.81	21.7±2.0**	83.8±5.6**	69.4±19**	80.2±2.6**
15.62	29.8±4.5**	84.4±4.7**	83.7±9.9**	82.1±5.2**
31.25	74.5±3.8**	84.9±4.4**	83.6±7.8**	82.0±4.9**
62.5	84.2±6.6**	84.9±4.3**	82.9±7.5**	80.0±5.7**
125	84.2±8.6**	84.4±3.3**	82.1±9.6**	77.7±6.3**
250	84.8±9.9**	84.6±2.9**	79.0±11**	71.5±4.8**
500	84.5±9.9**	84.1±4.3**	72.4±11**	59.4±4.4**

[1]% Cytotoxicity, as compared with untreated control (culture medium). Optical density for untreated control was 0.83 ± 0.006. Data represent mean ± SE of three replicate determinations from three independent experiments. Vehicle controls for hexane, methanol, chloroform, and methanol-aqueous partition were not cytotoxic for L5178Y-R cells; vincristine caused about 75% cytotoxicity to L5178Y-R cells at concentrations ranging from 0.24 to 125 µg·ml⁻¹. *$p < 0.05$, **$p < 0.01$, compared with untreated control.

Animals

Six-week-old female BALB/c mice (22 to 28 g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA). They were kept in a pathogen- and stress-free environment at 24°C, under a light-dark cycle (light phase, 06:00 to 18:00 h), and given water and food ad libitum.

Cell preparation and culture

Thymus and spleen were removed immediately after mouse death, and a single cell-suspension was prepared by disrupting the organs in RPMI 1640 medium as previously reported (Gomez-Flores et al., 2009). The cell suspensions were washed three times in this medium, suspended, and adjusted to 1×10^7 cells ml⁻¹ in complete RPMI 1640 medium.

T cell proliferation assay

T cell proliferation was determined by a colorimetric technique using MTT (Gomez-Flores et al., 2009). Thymus and spleen cell suspensions (100 µl of 1×10^7 cells ml⁻¹) were added to flat-bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 µl) of RPMI 1640 medium supplemented with 5% fetal bovine serum (unstimulated control), Con A (2.5 µg ml⁻¹), and plant extracts at various concentrations for 48 h at 37°C in 95% air-5% CO_2 atmosphere. After incubation for 44 h, MTT (0.5 mg/ml, final concentration) was added, and cultures were additionally incubated for 4 h. Cell cultures were then incubated for 16 h with extraction buffer (100 µl), and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (Becton Dickinson) at 570 nm. The lymphocyte proliferation index (LPI) was calculated as follows:

$$LPI = A_{570} \text{ in extract-treated cells/ } A_{570} \text{ in untreated cells}$$

Statistical analysis

The results were expressed as mean ± SE of three replicate determinations from three independent experiments. Statistical significance was assessed by the analysis of variance (ANOVA), $p < 0.05$, and pos-hoc Tukey, using statistical package for social sciences (SPSS) 21.

RESULTS

In vitro cytotoxic activity of *P. marginatus* extracts

The hexane, chloroform, and methanol extracts, respectively caused significant ($p < 0.05$) 20 to 85%, 32 to 84%, and 32 to 72% cytotoxicity to L5178Y-R cells at concentrations ranging from 1.9 to 500 µg ml⁻¹, respectively, and the methanol-aqueous partition caused significant ($p < 0.01$) 65 to 78% cytotoxicity at concentrations ranging from 3.9 to 125 µg ml⁻¹, respectively, and 72 and 59% cytotoxicity at 250 and 500 µg ml⁻¹, respectively, as compared with untreated control (Table 1). There was no statistical significance between extract treatment groups, as determined by ANOVA, $p <$

Table 2. Effect of *P. marginatus* extracts on B16F10 cells toxicity [%].

Concentration [µg ml^{-1}]	Hexane/vehicle	Chloroform/vehicle	Methanol/vehicle	Methanol-aqueous partition/vehicle
1.5	1	11.3±0.8	8.5±0.6	0
3.1	0	7.3±0.7	12.0±0.7	0
6.2	0	3.9±0.4	7.0±0.5	0
12.5	0	1.3±0.1	0	0
25	0	42.7±3.1**	0	39.6±3.9**
50	0	51.7±5.7**	44.4±5.2**	44.1±5.6**
100	39 ± 1.5**	50.7±4.9**	48.0±5.8**	41.9±5.9**

[1]% Cytotoxicity, as compared with untreated control (culture medium). Optical density for untreated control was 0.29 ± 0.01. Data represent mean ± SE of three replicate determinations from three independent experiments. Vehicle controls for hexane, methanol, chloroform, and methanol-aqueous partition were not cytotoxic for B16F10 cells; vincristine caused about 75% cytotoxicity to B16F10 cells at concentrations ranging from 0.24 to 125 µg·ml^{-1}. *$p < 0.05$, **$p < 0.01$, compared with untreated control.

Table 3. Effect of *P. marginatus* extracts on resident thymus/spleen cells proliferation [LPI].

Concentration [µg·ml^{-1}]	Hexane	Chloroform	Methanol	Methanol-aqueous partition
3.9	0.93/ND	0.85/ND	0.97/ND	0.9/ND
7.81	0.92[1]/1.08	0.8/1	0.94/0.95	0.8/0.98
15.62	0.90/1.07	0.8/1	0.91/0.96	0.7/1
31.25	0.96/1	0.8/1	0.9/0.96	0.9/1
62.5	0.94/1	0.81/0.95	0.9/0.95	0.7/1
125	0.89/0.5	0.81/0.84	0.92/0.98	0.6*/0.82

[1]LPI, as compared with untreated control (culture medium). Optical density for untreated control was 0.34 ± 0.002. Data represent mean ± SE of three replicate determinations from three independent experiments. Vehicle controls for hexane, methanol, chloroform, and methanol-aqueous partition were not cytotoxic for thymus and spleen cells (data not shown). *$p < 0.05$, compared with untreated control. ND, Not done.

0.05, and pos-hoc Tukey. With regards to B16F10 cells, the hexane, chloroform, methanol, and methanol-aqueous partition extracts, respectively caused significant ($p < 0.01$) 39% cytotoxicity at 100 µg ml^{-1}, 43 to 51% cytotoxicity at concentrations of 25 to 100 µg ml^{-1}, respectively, 44 to 48% cytotoxicity at concentrations of 25 to 100 µg ml^{-1} respectively, and 40 to 42% cytotoxicity at concentrations of 25 to 100 µg ml^{-1}, respectively (Table 2), as compared with untreated control. The hexane extract treatment caused the lowest cytotoxicity, whereas the other extract treatments were not significantly different from each other (ANOVA, $p < 0.05$, and pos-hoc Tukey). Vehicle controls for the extracts were not cytotoxic for L5178Y-R or B16F10 cells (data not shown). In addition, extracts were not cytotoxic for murine resident thymus and spleen cells (Table 3), except for the methanol-aqueous partition that caused significant ($p < 0.05$) 40% cytotoxicity to thymic cells at the concentration of 125 µg ml^{-1} (Table 3).

DISCUSSION

The pharmaceutical study and application of useful compounds from medicinal plants is very promising, particularly as an alternative to conventional cancer therapy (Tsuda et al., 2004). There are many reports on compounds with anticancer properties (Plaeger, 2003), including taxol, vincristine, vinblastine, tepocan, irinotecan, and etoposide-teniposide, which are commonly used in cancer therapy (Lu et al., 2003). The Cactaceae family is reported to contain about 1,500 species, which are distributed worldwide (Harlev et al., 2013). Medicinal uses of cacti include antitumor (Loro et al., 1999; Sreekanth et al., 2007; Franco-Molina et al., 2003; Harlev et al., 2013;), anti-inflammatory (Park et al., 1998; Loro et al., 1999), neuroprotective (Kim et al., 2006; Shetty et al., 2012), hepatoprotective (Ncibi et al., 2008), and anti-diabetes (Hassan et al., 2011) activities.

An important goal in medicinal plant research is to

avoid extensive and repetitive studies on known plants with limited anticancer potential, and focus on promising plants that have not been investigated; the present study was performed following these basic principles. We evaluated antitumor activity of *P. marginatus* crude extracts against murine lymphoma L5178Y-R and skin melanoma B16F10 cells, and demonstrated cytotoxicity with hexane, chloroform, methanol, and methanol-aqueous partition extracts. Cacti with antitumor potential include *Lophophora williamsii* (Lem. ex Salm-Dyck) Coult., also known as peyote, which was found to be cytotoxic to murine L5178Y-R and fibroblastoma L929, and human myeloid U937 and mammary gland MCF7 tumor cells (Franco-Molina et al., 2003); *Lophocereus schottii* (Engelm.) Britton & Rose has antitumor activity against murine lymphoma (Orozco-Barocio et al., 2013), and *Opuntia spp.* has been reported to have anti-tumor activity (Supino et al., 1996; Veronesi et al., 1999; De Palo et al., 2002; Zou et al., 2005).

The increasing resistance of mammalian tumor cells to chemotherapy, which causes adverse side effects, reduces its clinical efficacy. Thus, it is critical to discover and to develop novel anticancer agents from natural sources, such as plants, to overcome such resistance and side-effects. Cacti-based chemotherapy is a potential alternative to current cancer treatment. After applying *in vitro* bioreactor systems, valuable cacti metabolites could be produced for a large scale at limited costs and time (Lema-Rumińska and Kulus, 2014).

Conflict of Interests

The authors have not declared any conflict of interestS.

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