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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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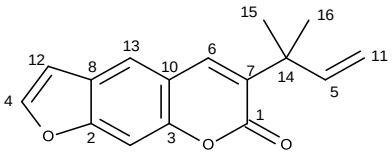

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$C_{16}H_{14}O_3$

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 10^3 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 ^{13}C-NMR spectrum of this molecule as the hydrogen and carbon connectivities in 2 were deduced from ^{1}H-^{1}H 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,

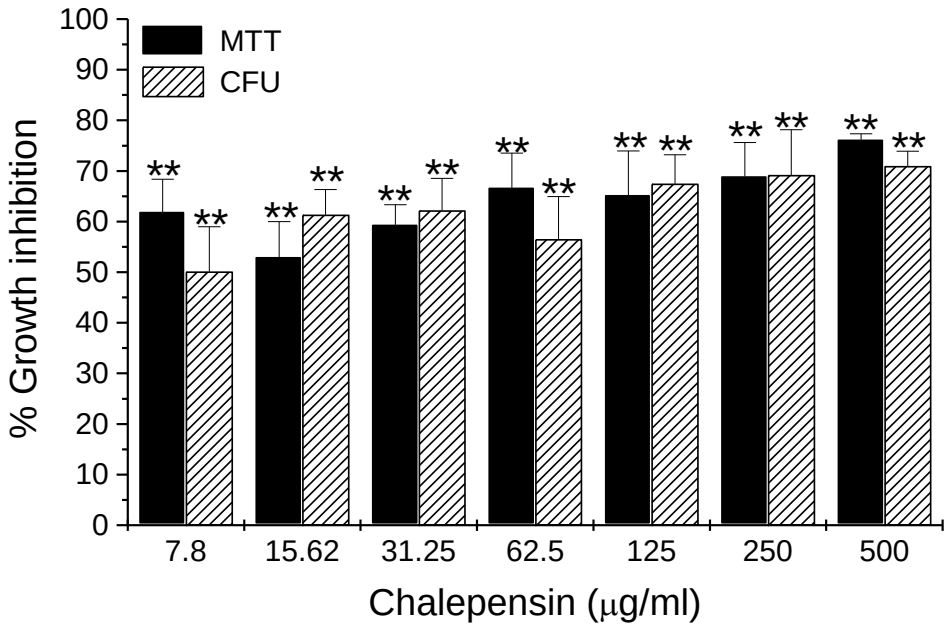

Figure 2. Antimicrobial effect of chalepensin from *R. chalepensis* on *S. mutans* growth. *S. mutans* culture suspensions were incubated with chalepensin, followed by measuring viability by the MTT reduction and CFU methods, as explained in the text. Data indicate the mean ± SE of 3 replicate determinations 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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