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Antimicrobial and cytotoxic activities of *Barleria prionitis* and *Barleria grandiflora*: A comparative study

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Abstract

Leaf juices as well as leaves of *Barleria prionitis* and *Barleria grandiflora* are being used by rural people across various regions of India in the treatment of oral ailments such as dental troubles, gum ailments, pyorrhoea, dental carries and mouth ulcers. Zone of inhibition and MIC values obtained for all the extracts suggest ethanolic extract of the herbs were more antimicrobial when compared to the aqueous extract. Results of biofilm suppression were found statistically significant ($p<0.05$) when compared to control. Cytotoxicity was evaluated by MTT assay on human gingival fibroblast and human dermal fibroblast cell lines for ethanolic extract of the herbs. CTC_{50} value was found to be more than 1,000 µg/mL for ethanolic extracts of both herbs. Chlorhexidine was found to be more cytotoxic with CTC_{50} value of 12.5–25 µg/mL. Ethanolic extract of *B. prionitis* and *B. grandiflora* found significantly cytotoxic ($p<0.05$) in comparison with control.

Introduction

Alternative system of medicine largely comprises of the use of plants and plant derived products (Banerjee et al., 2012; Singh and Dubey, 2012; Rawat et al., 2010). Genus *Barleria* L. belonging to family acanthaceae largely comprises of more than 300 species of shrubs and herbs. These species are mainly found in Asia and Africa (Balkwill and Balkwill, 1998). India is represented by 26 to 32 species, one subspecies and one variety (Shendage and Yadav, 2010; Karthikeyan et al., 2009; Balkwill and Balkwill, 1997).

Barleria prionitis, commonly known as vajradanti, has been used for treatment of various ailments by rural population across India. Some uses of the plant and various parts of the plants are in cases of asthma, whooping-cough, rheumatism, cough ailment, fever, infection related ailments, neuralgia, snakebite, liver ailments, piles, ulcers, irritation control, wound healing, dropsy, liver congestion, cataract, boils, glandular swellings, stiffness of limbs, sciatica, enlargement of scrotum, increasing vigor, gout, edema, malaria, leucoderma, scabies etc. (Rani and Kumar, 2015; Sharma et al., 2013; Banerjee et al., 2012). Leaves of *B. prionitis* are chewed or juice of the leaves are used against toothache, gum ailments, dental troubles, pyorrhea and mouth ulcers (Katewa and Galav, 2005; Mahajan, 2007; Sankaranarayanan et al., 2010; Reddy et al., 2010; Singh and Dubey, 2012).

B. grandiflora Dalz is an another species of this genus, leaves of which are being chewed by rural population across central India for the treatment of mouth ulcer, stomatitis and gingivitis (Sawarkar et al., 2009; Salunkhe et al., 2013; Jayanthi et al., 2014). Literature survey reveals that although these plants and their extracts evaluated for a number of pharmacological activities, not much of the work has been reported towards their usefulness in oral ailment.

The present study aimed to compare the usefulness of leaf extracts of *B. prionitis* and *B. grandiflora* in treatment of oral ailment like aphthous ulcers.

Materials and Methods

Plant material

The herbs had been collected in October, 2014 (flowering stage) from a rural region of Amravati district of Maharashtra (India). The herbs had been identified and authenticated by Prof. Ranjana Mishra, Department of Botany, Durg Science College, Durg (Chhattisgarh). Latter, leaves of *B. prionitis* and *B. grandiflora were* collected in February, 2015 (fruiting stage), dried in shade, powdered and used for extraction.

Extraction of plant material

The powdered plant materials had been treated with petroleum ether for defatting. Plant materials, thus, obtained further subjected to hot continuous extraction and cold maceration to get ethanolic extracts and aqueous extracts respectively. All the four extracts, then, treated with dichloromethane and ethyl acetate subsequently; in order achieve complete removal of fatty material from the plant material and designated as ethanolic extract of *B. grandiflora*, ethanolic extract of *B. prionitis*, aqueous extract of *B. grandiflora* and aqueous extract of *B. prionitis*.

Chemicals and reagents

Petroleum ether, dichloromethane, ethyl acetate, acetic acid, crystal violet dye, dimethyl sulfoxide (DMSO) all chemicals from Molychem. Ethanol was purchased from Changshu Hong-sheng Fine Chemicals. Resazurin, nutrient blood agar media, Sabouraud dextrose agar medium, Mueller Hinton broth, Sabouraud dextrose broth were procured from HiMedia. Marketed preparation of positive standard chlorhexidine had been purchased from local chemist at Raipur. Dulbeccos modified Eagle medium, fetal bovine serum, serum free media and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, all from Sigma-Aldrich had been used under facilities provided at IVM and Microbiology Center, Himalaya Drug Company (India).

Microorganism and growth conditions

Bacterial cultures *Staphylococcus aureus* MTCC 3160, *Streptococcus* mutans MTCC 890 and fungal culture *Candida albicans* MTCC 3017 were procured from Imtech, India. *Lactobacillus sporogens* [Sporlac powder, Sanzyme (P) Ltd.] purchased from local chemist. Bacterial strain had been cultured in nutrient blood agar media and fungal strain in Sabouraud dextrose

Box 1: Biofilm suppression

Principle

Biofilm suppression determined by microtiter biofilm formation assay is a qualitative and quantitative method to study the biofilms. Biofilms are large colonies of micro-organisms surrounded by fluid-filled channels and regarded as the one of the major factors responsible for antibiotic tolerance and resistance. Staining agent crystal violet, will impart violet coloration to the biofilms formed during the assay. In presence of antimicrobial component, the violet coloration will be lesser when compared to control, which can be quantified by reading absorbance values of the test solutions and standards using microcuvetes.

Requirements

S. aureus MTCC 3160, *S. mutans* MTCC 890, *C. albicans* 3017, *L. sporogens* (Sporlac powder), Mueller Hinton broth, Sabouraud dextrose broth, ethanolic extract of *B. prionitis* and *B. grandiflora*, marketed preparation of chlorhexidine, dimethyl sulfoxide, microtiter plate, 0.1% crystal violet dye, 30% acetic acid, incubator and double beam spectrophotometer (UV-1800, Shimadzu)

Procedure

Step 1: The overnight cultures bacteria and the fungus had been diluted to 1:100 respectively in Mueller Hinton broth and Sabouraud dextrose broth. Each well, seeded with 100 µL of bacterial and fungal cultures. The microtitre plate had been incubated at 37°C for 24 hours.

Step 2: Two gentle washings were given by sterile distilled water to remove bacterial and fungal cells on the surface or unbound cells.

Step 3: All the test extracts were reconstituted in dimethyl sulfoxide and diluted in respective broth to obtain concentration of 5 mg/mL. 100 µL of each of the extracts was then added in to the wells and incubated at 37°C for 4 hours. Similarly, 100 µL of chlorhexidine (50 µg/mL) was added to the wells and incubated at 37°C for 4 hours. 100 µL of broths were added to the control wells and incubated at 37°C for 4 hours.

Step 4: Contents of the wells were discarded after incubation.

Step 5: 100 µL of staining agent (0.1% crystal violet) was added to each well and incubation had been carried out at room temperature for 15 min.

Step 6: Sterile distilled water was used to remove excess of staining. Three washings were given with sterile distilled water and plates were then air dried.

Step 7: 125 µL of acetic acid (30%) was used for the destaining of microbial cells. The destaining was carried out for 15 min.

Step 8: Quantification of biofilm was done by transferring the contents of the wells to another microtiter plate and by taking the absorbance readings at 600 nm using spectrophotometer.

Step 9: Percentage inhibition of the biofilm was determined by using the formula,

Percentage inhibition = 100 - {(Sample Absorbance/ Control Absorbance) × 100}

Where, Sample Absorbance means absorbance observed with EBP, EBG and chlorhexidine separately. Control Absorbance means absorbance observed with 30% acetic acid in water.

References

O'Toole, 2011

Anand et al., 2015

Box 2: Cytotoxicity assay

Principle

MTT assay is an *in vitro* method to study cytotoxicity. The method evaluates the cellular metabolic activity resulting into formation of dark purple colored formazan. MTT is converted into formazan due cellular mitochondrial dehydrogenase enzyme.

Requirement

Human gingival fibroblast cell lines, human dermal fibroblast cell lines, DMEM, FBS, serum free media, test extracts, chlorhexidine, DMSO, 96-well microtiter plates, PBS, double beam UV-spectrophotometer (UV-1800, Shimadzu).

Procedure

Step 1: Cells had been seeded (1×10^4 cells/mL) in 96-well plates in DMEM with high glucose and 10% FBS. Then , these were incubated for 24 hours at 37°C with 5% CO_2.

Step 2: The initial stock solution of test extracts (10 mg/mL) was prepared by dissolving in DMSO and subsequent dilutions had been made in serum free medium.

Step 3: The extracts were diluted for obtaining the concentrations of 1000, 500, 250, 125, 62.5, 31.2 and 15.6 µg/mL. The marketed preparation of chlorhexidine was diluted in serum free medium to obtain concentrations of 100, 50, 25, 12.5, 6.25, 3.1, 1.5 µg/mL.

Step 4: The dilutions (100 µL/ well) added to cells. The plates had been further incubated at 37.1°C with 2.1% CO_2 and relative humidity of 44.4%. Cell control (prepared by mixing equal volumes of DMSO and serum free media) had also been maintained.

Step 5: After 24 hours incubation, the morphological changes of the cells were observed. The cytotoxicity was determined by MTT assay. At this stage, the content of the wells was removed gently and 100 µL of MTT solution was added to each well.

Step 6: The plate was incubated for 4 hours at 37.1°C with 2.1% of CO_2 and relative humidity of 44.4%.

Step 7: After incubation, the cell supernatant was removed and cell monolayer was washed with PBS twice.

Step 8: Finally, 100 µL of DMSO was added to each well to extract out the cell bound dye and incubation was carried out for 15 min. The absorbance was measured using spectrophotometer at 540 nm.

Step 9: The % cytotoxicity was calculated from absorbance values of treated and control groups. The CTC_{50} values for the sample were calculated from the dose response curves by linear regression analysis.

The percent cytotoxicity was calculated by using formula:

Percent cytotoxicity = [(C1- T1)/ C1] × 100

Where, C1 = Absorbance of cell control group and T1 = Absorbance of the test

References

Avila and Pugsley, 2011

Mossman, 1983

Rodanant et al., 2012

agar medium. The subcultures of bacterial strains had been prepared in nutrient broth for agar well diffusion assay and for biofilm suppression assay, the subcultures prepared in Mueller Hinton broth. The subculture of *C. albicans* had been prepared in Sabouraud dextrose broth. The bacterial and fungal subcultures were incubated at 37°C, for 24 hours and used for antimicrobial and biofilm suppression activities.

Cell lines

Human gingival fibroblast and human dermal fibroblast cell lines had been used for cytotoxicity study. DMEM supplemented with 15% FBS, streptomycin (100 µg/mL), penicillin (100 U/mL) and amphotericin B (0.25 mg/mL) was used for maintaining the cell lines. Both the cell lines maintained at 37.1°C with 2.1% CO_2 and relative humidity of 44.4% in an incubator.

Antimicrobial activity

The antimicrobial activity against bacterial and fungal strains was performed for comparing the effectiveness of the extracts of both the herbs. The antimicrobial activity of the extracts had been evaluated against oral infectious microbial strains, *S. aureus*, *S. mutans*, *L. sporogens* and *C. albicans* by agar well diffusion method (Ahmad et al., 1998; Perumalsamy and Ignacimuthu, 2000; Werner et al., 1999). The bacterial cultures and fungal culture had been prepared in nutrient broth agar and Sabouraud dextrose agar respectively by pouring sufficient amount of strains aseptically. The sterile borer (8 mm) was used to make wells in petri plates. All the extracts were mixed in DMSO for obtaining final concentration of 5 mg/mL. The wells had been filled with 1 mL of extracts and compared with chlorhexidine (50 µg/mL) for antimicrobial potential. All the plates were incubated for 24 hours at 37°C. After 24 hours of incubation the zone of inhibition measured in mm. DMSO had been used as control.

Determination of minimum inhibitory concentration (MIC)

Microdilution method with slight modifications (Sarker et al., 2007) was used for determining the MIC for the extracts, chlorhexidine and control. DMSO was used for diluting the extracts and chlorhexidine. All the extracts were diluted further with sterile normal saline for obtaining serial dilutions as 5000, 2500, 1250, 625, 312.5, 106.2, 53.1 and 26.5 µg/mL. The positive standard chlorhexidine was diluted for obtaining serial dilutions as 50, 25, 12.5, 6.2, 3.1, 1.0, 0.5 and 0.2 µg/mL. The control was prepared using DMSO and sterile normal

saline. The row of wells designated for extracts and standard had been pipetted with 240 µL of the extracts and chlorhexidine. Similarly, 240 µL of control had also been pipetted in to designated wells. These wells had been added with 10 µL of resazurin indicator solution. Preparation of resazurin indicator solution was done by dissolving 270 mg of resazurin in 40 mL of distilled water. Further, 30 µL of nutrient broth and Sabouraud dextrose broth had been pipetted into the wells with respect to the microbial culture to be inoculated. Finally, 10 µL of bacterial or fungal culture had been added to each well. The plates then incubated for 24 hours at 37°C. The MIC taken as the minimum concentration at which color changes from purple to pink or colorless.

Statistical analysis

All the results had been expressed as mean ± standard deviation. Results of biofilm suppression and cytotoxicity were further analyzed using one-way ANOVA followed by Dunnett multiple comparison test.

Results

Antimicrobial activity and determination of MIC

The result of antimicrobial activity suggested the effectiveness of ethanolic extracts when compared to aqueous extracts of both herbs. All the extracts except ethanolic extract of *B. prionitis* (same MIC for bacterial and fungal strains) found to be more antibacterial (lower MIC) than antifungal. The antimicrobial activity of the extracts found to be less than the chlorhexidine (Table I). The antimicrobial activity of all the extracts was statistically significant ($p<0.05$) in comparison to chlorhexidine. Among the extracts, ethanolic extract of *B. prionitis* observed to be most effective against test microbes followed by the extract of *B. grandiflora*. *B. prionitis* shown same antibacterial and antifungal potential (MIC value in between 53.1–106.2 µg/mL for all test microbes. Extract of *B. prionitis* showed significant antimicrobial potential with MIC value in between 106.2-312.5 µg/mL for bacterial strain and MIC value of 312.5-625 µg/mL for fungal strain.

Table I

Antimicrobial activity of leaf extracts

Sample	Concentration (µg/mL)	Zone of inhibition and MIC							
		S. mutans	MIC (µg/mL)	*S. aureus*	MIC (µg/mL)	*L. sporogens*	MIC (µg/mL)	*C. albicans*	MIC (µg/mL)
B. grandiflora (aqueous)*	5000	13.5 (0.1)	625- 1250	16.6 (0.0)	625-1250	16.3 (0.0)	625- 1250	17.1 (0.1)	1250-2500
B. grandiflora (ethanol)*	5000	21.1 (0.1)	106.2-312.5	19.7 (0.1)	106.2-312.5	20.2 (0.1)	106.2- 312.5	22.6 (0.0)	312.5-625
B. prionitis (aqueous)*	5000	23.1 (0.1)	312.5- 625	19.2 (0.1)	312.5-625	16.3 (0.1)	312.5- 625	19.3 (0.1)	312.5-625
B. prionitis (ethanol)*	5000	25.9 (0.0)	53.1-106.2	24.6 (0.1)	53.1-106.2	25.5 (0.1)	53.1-106.2	26.6 (0.1)	53.1-106.2
Chlorhexidine	50	28.6 (0.1)	3.1-6.2	29.4 (0.1)	3.1-6.2	29.6 (0.0)	3.1-6.2	28.1 (0.3)	3.1-6.2
Dimethyl sulfoxide	-	0	0	0	0	0	0	0	0

*Indicates statistically significant ($p<0.05$) antimicrobial activity of extracts in comparison to chlorhexidine; Data are mean; SD are within parenthesis

Table II

%Biofilm suppression

Test organism	*B. prionitis* (ethanol extract)	*B. grandiflora* (ethanol extract)	Chlorhexidine
S. aureus	85.1 ± 1.8•	79.7 ± 2.3*	87.8 ± 2.1
S. mutans	86.9 ± 1.1•	81.6 ± 2.5*	90.1 ± 2.3
L. sporogens	90.5 ± 1.9•	85.7 ± 1.6*	91.8 ± 1.1
C. albicans	86.0 ± 3.6•	80.5 ± 2.4•	83.0± 3.3

*Indicates statistical significance ($p<0.05$) of EBG and • indicates statistical non significance of EBP and EBG ($p>0.05$) in biofilm suppression as compared to chlorhexidine

Biofilm suppression

Ethanolic extract of *B. prionitis* and *B. grandiflora* were significantly suppressing the biofilms of test organisms ($p<0.05$) when compared to control (DMSO) at tested concentrations (Table II). Particularly ethanolic extract of *B. prionitis* proved to be more effective than ethanolic extract of *B. grandiflora* in biofilm suppression but results were not statistically significant ($p>0.05$) when compared to chlorhexidine. There was no significant difference observed between the ethanolic extract of *B. prionitis* and chlorhexidine at tested concentrations. Biofilm suppression with ethanolic extract of *B. grandiflora* observed statistically significant ($p<0.05$) when compared to standard particularly against all test bacterial strains. Ethanolic extract of *B. prionitis* and *B. grandiflora* both at tested concentration found no significant ($p>0.05$) in biofilm suppression against *C. albicans*.

Cytotoxicity assay by MTT

Cytotoxic effect of the ethanolic extract of *B. prionitis* and *B. grandiflora* on human gingival fibroblast cells were assessed using MTT assay. Based on the results of antimicrobial effects only ethanolic extracts were subjected for evaluation of cytotoxicity. The results had shown ethanolic extract of *B. prionitis* and *B. Grandiflora* at >1000 µg/mL caused 50% cytotoxicity (CTC_{50}) to human gingival fibroblast cell lines, human dermal fibroblast cell lines. CTC_{50} value for chlorhexidine was found to be 12.5-25 µg/mL with human gingival fibroblast cell line. CTC_{50} value for chlorhexidine found to be 25 µg/mL with human dermal fibroblast cell line (Figure 1). Statistically both *B. prionitis* and *B. grandiflora* were found significant when compared to control ($p<0.05$).

Discussion

The result of the study showed the *in vitro* effectiveness of ethanolic extracts of both the herbs. Ethanolic extract of *B. prionitis* was most effective among all the extracts with MIC of 53.1-106.2 µg/mL against all tested microbes. MIC for *B. grandiflora* was found 106.2-312.5 µg/ mL (bacterial strains) and 312.5-625 µg/ mL (fungal strain). Hence, for further study ethanolic extracts had been taken. The extracts evaluated for biofilm suppression and results found at par with chlorhexidine having no significant difference. Ethanolic extract of *B. prionitis* was found to be showing higher antifungal potential than standard at tested concentrations. Cytotoxicity assessed by MTT assay and CTC_{50} values for ethanolic extract of *B. prionitis* and ethanolic extract of *B. grandiflora* observed to be more than 1000 µg/mL.

Kosmulalage et al. (2007) reported strong antibacterial activity of ethanolic extract and some phytochemicals like balarenone, pipataline, 13, 14- stigmasta-5-14-dione -3-ol, isolated from ethanolic extract of *B. prionitis*. Aneja et al. (2010) showed the potent antifungal and antibacterial activity of methanolic extract of *B. prionitis* bark against bacteria and fungi involved in oral diseases of human. Kumar et al. (2013) reported antibacterial activity in methanolic extract of the leaves of *B. prionitis*. It was shown higher antibacterial potential of ethyl acetate extract of *B. prionitis* leaves against Gram positive strains of bacteria than Gram negative strains. We report the similar kind of antimicrobial potential of ethanolic extract of *B. prionitis* leaves with different MIC values. In our study, the MIC for ethanolic extract of *B. prionitis* was observed as 53.1-106.2 µg/mL against all test microbes. In our earlier study, we have reported the anti-oxidant potential of ethanolic, hydroalcoholic and aqueous extracts of *B. grandiflora* leaves (Sawarkar et al., 2009; Nishant et al., 2014).

Kumari et al., (2015) reported the antifungal potential of aqueous extract of *B. grandiflora*. We are, for the first time, reporting antibacterial as well as antifungal potential of the extracts obtained from the leaves of *B. grandiflora*. We found significant antimicrobial potential of ethanolic extract of *B. grandiflora* with MIC value of 106.2-312.5 µg/mL and 312.5-625 µg/mL for bacterial strains and fungal strain under study. Our result differs for antifungal activity than the results reported by the authors earlier. We observed more potent activity in ethanolic extracts against *C. albicans*; however, the fungal strain used by the authors earlier was *Aspergillus fumigatus*. We are reporting for the first time the cytotoxic potential and biofilm suppressing potential of *B. prionitis* and *B. grandiflora*. In comparison to control, ethanolic extract of *B. prionitis* and ethanolic extract of *B. grandiflora* found cytotoxic on the other hand both the extracts found less cytotoxic when compared to chlorhexidine.

Therapeutic treatments of aphthous ulcer include use of anti-inflammatory agents, immunomodulatory drugs, antibiotics, anti-oxidant and others. The study was undertaken to authenticate the use of *B. prionitis* and *B. grandiflora* in treatment of oral ailments such as gingivitis, stomatitis and mouth ulcer by rural people across some regions of India. Oral infections such as dental carries, periodontal diseases, aphthous ulcers and peri implant diseases are known to cause by some microbes like *Enterococcus faecalis*, *S. aureus*, *S. mutans*, *Escherichia coli* and *C. albicans* due to the formation of biofilm (Filoche et al., 2010).

The therapeutic efficacy of the herbs is attributed to various chemical constituents present in them. *B. prionitis* is reported to have phytochemicals like glycosides, anthraquinone, saponins, flavanoids and phenolic compounds.

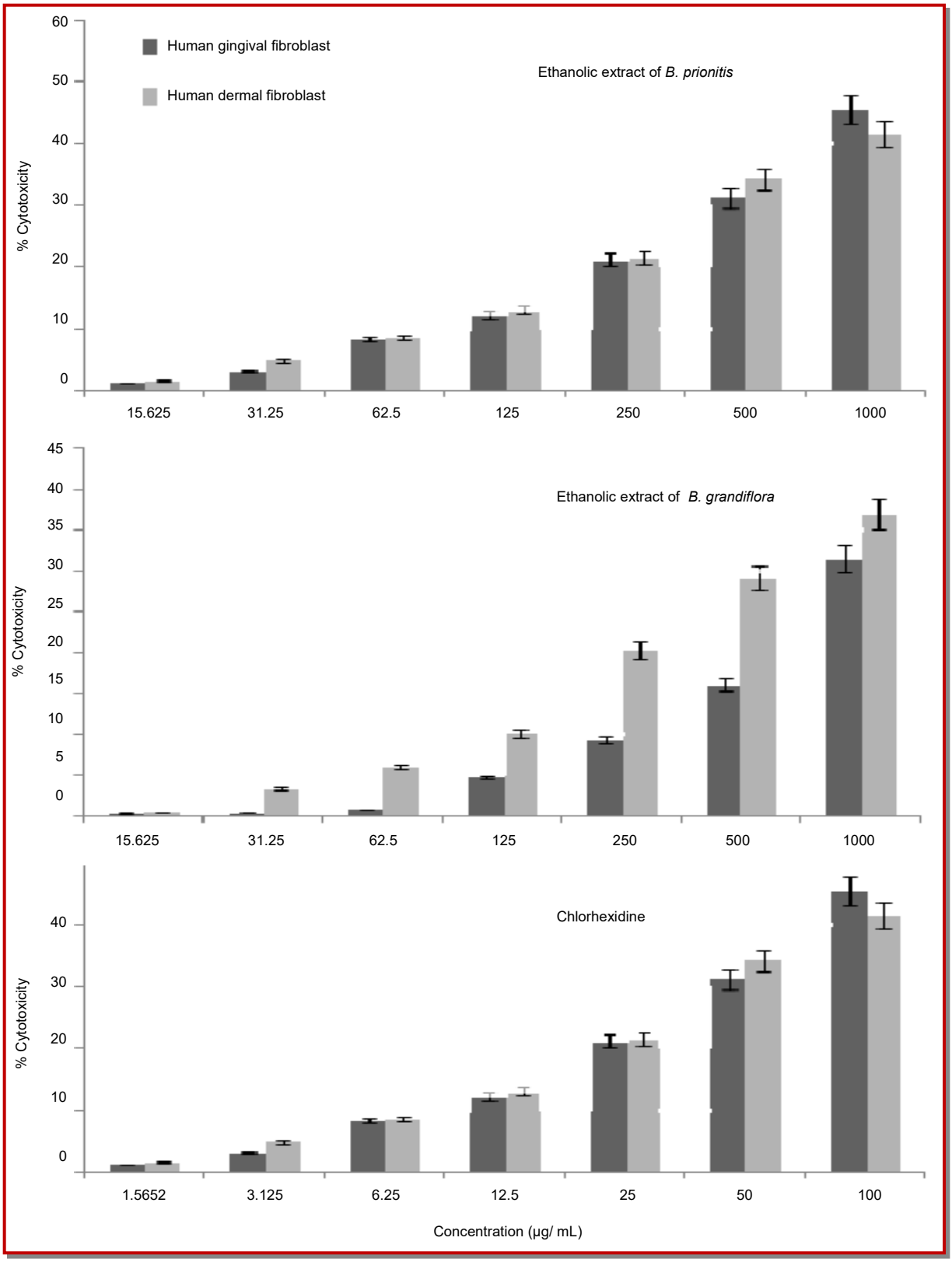

Figure 1: Cytotoxicity activity of the ethanolic extract of *B. prionitis* (A), *B. grandiflora* (B) and chlorhexidine (C) on the human gingival fibroblast and human dermal fibroblast cell lines by MTT assay

Some of the isolated compound from the herbs to name few are, 6-hydroxyflavones, scutellarein-7-rhamnosyl glucoside, barlerin, acetyl barlerin, luteolin-7-O-β-D-glucoside, shanziside methyl ester, 6-O-trans-p-coumaroyl-8-O-acetyl shanziside methyl ester, lupilinoside7-methoxydiderroside, 1,8 dihydroxy-2,7-dimethyl-3,6-dimethoxy anthraquinone,1,3,6,8-tetramethoxy-2,7-dimethyl anthraquinone, prioniside A, prioniside B, prioniside C, balarenone, pipataline (Taneja and Tiwari, 1975; Soren et al., 1982; Gupta and Saxena; 1984; Chen et al., 1998; Singh et al., 2005; Ata et al., 2009; Ganga Raju et al., 2002; Ata et al., 2007; Kosmulalage et al., 2007).

B. grandiflora has been reported to have phytochemicals like glycosides, anthraquonone, saponins, flavanoids and phenolic compounds (Sawarkar et al., 2009). *B. grandiflora* yet not been investigated for the isolates. It is a well established fact that biological activities of the crude drugs are due to phytochemicals present in it. Thus it would be a possibility that the antimicrobial activity, biofilm suppression and cytotoxic potential of these herbs may be due to one or more phytochemicals present in them.

Conclusion

The usefulness of *B. prionitis* and *B. grandiflora* in treatment of oral ailments may be due to their antimicrobial and cytotoxic potential. Ethanolic extracts of both the herbs were found to be having more antimicrobial potential than to that of aqueous extracts and found less cytotoxic than chlorhexidine.

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Conflict of Interest

Authors declare no conflict of interest

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