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Invitro Investigation for Minimum Inhibitory Concentration (MIC) And Cytotoxicity Assay of Bromelain-Quercetin

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Abstract

The application of herbal remedies (Phytotherapy) and products for the treatment of gingivitis, periodontitis, and dental caries has gained significant popularity among dental professionals and patients alike. Due to their alkaline nature, the antibacterial properties of medicinal plants help to prevent the formation of plaque and calculus by maintaining the acid-alkali balance in saliva. Herbal medicine includes herbs, plant-derived materials, preparations and products that utilize plant parts or other botanical substances as their active ingredients [1]. Bromelain is one such substance, a crude extract derived from pineapple, which includes, among other substances, several closely related proteinases. These proteinases demonstrate various activities, including fibrinolytic, anti-oedematous, anti-inflammatory and antithrombotic, effects both in vitro and in vivo.

Keywords

Invitro Investigation; Minimum inhibitory concentration; Cytotoxicity assay; Bromelain-quercetin.

Introduction

The application of herbal remedies (Phytotherapy) and products for the treatment of gingivitis, periodontitis, and dental caries has gained significant popularity among dental professionals and patients alike. Due to their alkaline nature, the antibacterial properties of medicinal plants help to prevent the formation of plaque and calculus by maintaining the acid-alkali balance in saliva. Herbal medicine includes herbs, plant-derived materials, preparations and products that utilize plant parts or other botanical substances as their active ingredients [1]. Bromelain is one such substance, a crude extract derived from pineapple, which includes, among other substances, several closely related proteinases. These proteinases demonstrate various activities, including fibrinolytic, anti-oedematous, anti-inflammatory and antithrombotic, effects both in vitro and in vivo.

Bromelain has been recognized chemically since 1875 and is utilized as a phytochemical compound. It is regarded as a dietary supplement and is readily accessible to the public in health food stores and pharmacies across the USA and Europe [4]. Current research suggests that bromelain may serve as a potential candidate for the advancement of future oral enzyme therapies aimed at oncology patients. Bromelain is capable of being absorbed in the human intestines without undergoing degradation and retains its biological activity [7]. Quercetin is also a type of dietary flavonoid found in various fruits and vegetables, including cherries, broccoli, red onions, and mangoes. Recent studies have demonstrated that quercetin possesses significant pharmaceutical properties owing to its antioxidant, anti-inflammatory, antimicrobial, antineoplastic, neuroprotective, and antiallergic effect supports its categorization as a nutraceutical and validate its examination in chronic diseases linked to oxidative stress. There are various applications of quercetin along with the treatment of oral diseases [8]. Numerous systematic reviews have demonstrated the antineoplastic effects of quercetin in vitro. The present study aims to 'Investigate the Minimal inhibitory concentration and Cytotoxic effect of the combination of Bromelain-quercetin- An In-vitro study.

Materials And Methods

This analytical study was conducted in Krishnadevaraya Dental College and Hospital (KCDSH), Bangalore, Karnataka. Patients presenting with Stage II/III and Grade B periodontitis were enrolled for collection of Gingival Crevicular Fluid (GCF) using Paper points (Dia Dent), ® from Out Outpatient Department (OPD) of Periodontology and investigation of the Bromelain-quercetin was performed at Department of Microbiology, KCDSH, Bangalore, Karnataka.

Gingival crevicular fluid (GCF) sample collection was done at the (OPD) of Periodontology, to check the Minimal inhibitory concentration (MIC). Cytotoxic effect of bromelain-quercetin was done on human gingival fibroblast by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay at Stroma Biotechnologies Private limited, Yeshwanthpur Bangalore.

Minimum inhibitory concentration assay (MIC)

MIC by tube (broth) dilution was determined by exposing a standardized inoculum to a series of two-fold drug concentrations in tubes of broth and finding the lowest concentration with no visible growth.

Reagents and materials

- Thioglycollate broth with Hemin & Vitamin K (HiMedia, Mumbai)
- Bromelain – Quercetin powder of known potency to prepare stock solution
- Sterile 2 mL Eppendorf tubes, Micropipette, sterile microtips.
- 0.85% saline for preparing bacterial inoculum.
- 0.5 McFarland standard and device/background card for turbidity adjustment.
- Test organism grown on Thioglycollate broth with Hemin & Vitamin K for 18–24h.

Procedure for MIC estimation

Step 1: Prepare antibiotic stock and dilution series (Table 1)

1. Calculate amount of antimicrobial powder required to prepare a stock solution at 8%
2. Dissolve in the recommended diluent (Distilled water /DMSO) to the calculated concentration; mix until fully dissolved and label with drug name, concentration, date, and storage conditions.
3. For macrodilution, plan a series of two-fold dilutions covering the expected clinical range 0.0156, 0.0312, 0.062, 0.125, 0.25, 0.5, 1, 2, 4, 8 mg/mL final concentrations in tubes).
4. Dispense 1 mL of BHI broth into each sterile tube; add appropriate volumes of stock and broth to prepare a set of two-fold concentrations at 2× the desired final concentration (because inoculum will dilute 1:2 when added).

Example (for 1 mL volume before inoculation):

- Tube 1: highest concentration (e.g. 16 µg/mL in broth; will become 8 µg/mL after inoculation).
- Perform serial two-fold dilutions by transferring equal volumes (e.g. 0.5 mL) from one tube to the next, adding to 0.5 mL broth, mix well, and continue through all tubes; discard 0.5 mL from last tube to equalize volume. (Figure 1)

1. Include one positive growth control tube with broth only (no antibiotic) and one sterility control with broth only and no inoculum.
2. **Step 2:** Prepare standardized inoculum
3. From an 18–24 h culture on Thioglycolates broth with Hemin & Vitamin K to achieve near 0.5 McFarland turbidity (approx. 1.5×10^8 CFU/mL).
4. Compare visually with a 0.5 McFarland standard against a white card with black lines; adjust by adding more culture (to increase turbidity) or more broth (to decrease).
5. Dilute the 0.5 McFarland adjusted suspension 1:150 in broth to obtain about 1×10^6 CFU/mL, then mixing 0.5 mL of this with 0.5 mL drug solution in each tube leads to 5×10^5 CFU/mL final inoculum.

Step 3: Inoculation of tubes

1. Label all tubes with antibiotic concentration, organism ID, and date
2. Within 15 minutes after standardizing the inoculum, add an equal volume of inoculum (0.5 mL) to each tube containing 0.5 mL of antibiotic dilution, including the growth control tube (broth only).

3. Do not inoculate the sterility control tube.
4. Mix gently (by tapping or vortexing briefly) to distribute organisms uniformly without causing foaming.

At this point each test tube contains: Defined antibiotic concentration (halved from initial 2× dilution).

Final inoculum will be of approximately 5 x 105 CFU/mL.

Step 4: Incubation

- Incubate tubes at 37 °C in anaerobic jar for 16–20h

Step 5: Reading and interpretation

1. After incubation, the optical density of the growth in all tubes will be measured in spectrophotometer at 600 nm. (Figure 2)
2. MIC is the lowest drug concentration whose OD_{600} indicates no growth compared with the inoculated, drug-free control.

	Serial doubling dilution of BQ										Growth control	Sterility control
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	Tube 10	Tube 11	Tube 12
Broth	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml
BQ	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	-	-
Conc	8%	4%	2%	1%	0.5%	0.25%	0.012	0.0062	0.0312	0.0156	-	-
Culture	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml	-

Table 1: Serial doubling dilution of BQ.

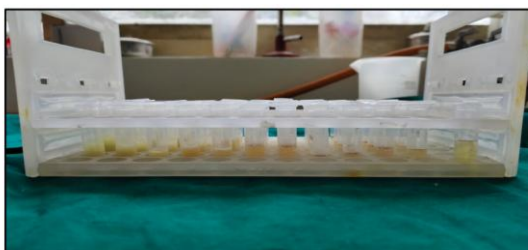
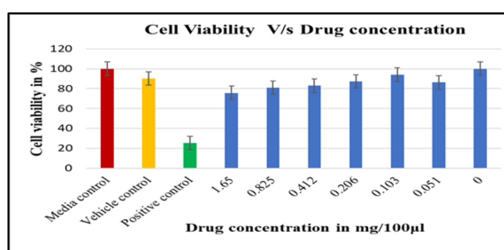


Figure 1: Photographic view of MIC done in tube dilution method.

Figure 2: Photographic view of optical density of the growth seen in test tubes will be measured in spectrophotometer at 600nm.

Cell viability determined using the MTT Assay (Figure 3 and Figure 4, Graph 1)

(3- [4, 5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide). Cells were seeded in a fibronectin-coated 24 x 12 well plate for cultivation at 37 °C. After 48 h, 20 mL of MTT 5g/L (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to each well at a final concentration of 0.5mg/ml after 4 hours of shock wave treatment. Cells were cultured in a 24 x 12 well plate to 70% confluency and treated with shock wave pulses as described above. Cells were incubated in MTT medium for 3 hours at 37 °C in the dark, and formazan crystals were solubilized using 50% Dimethyl Sulfoxide (DMSO). Absorbance was read at 570 nm using Eppendorf Bio-spectrometer with DMSO as reference. The test sample induced 50% cell death in Human fibroblast cells at a concentration **of 55.78mg.**



Graph 1: Sample concentration vs. cell viability.

Figure 3: Human Gingival fibroblasts cells seeded in a 96 well plate (2000 cells/well).

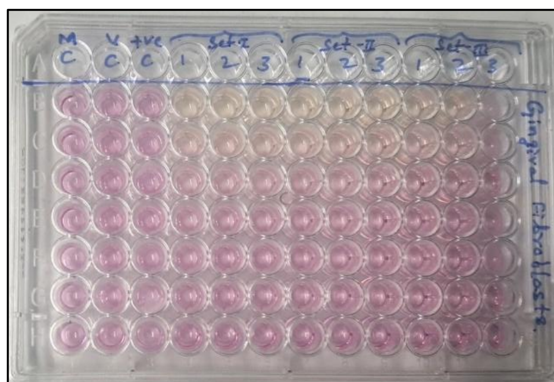


Figure 4: 96-well plate with test compound (drug).

Results

The effective concentration was followed by the determination of cell viability-MTT Assay. (Stroma Lifesciences Pvt Ltd). The test sample induced 50% cell death in Human fibroblast cells at a concentration of 55.78 mg. Minimum inhibitory concentration (MIC) of bromelain and quercetin is the lowest drug concentration whose OD_{600} indicates no growth compared with the inoculated of 0.25% concentration with mean reading 0.214 with colony forming unit of 0.401*108 CFU/ml.

Discussion

Taherh Kerdar et al. conducted a randomized controlled trial involving 50 patients suffering from chronic periodontitis, who were randomly assigned to two groups: the experimental group utilized a mouthwash made from Xuanshen herbal extract (which contains quercetin), while the control group used a commercial mouthwash. The findings indicated that the mouthwash with quercetin was effective in improving the plaque index (PI), periodontal pocket depth (PD), and bleeding on probing (BOP). Cheng et al. created animal models of periodontitis and found that the average bone resorption was significantly lower at 8 and 12 days in the quercetin group. Comparable results were also observed in another study [6].

In the treatment of periodontitis, quercetin demonstrated significant effectiveness against two major periodontal pathogens, Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans [6]. A prior study applied various concentrations of quercetin to these pathogens and revealed a noticeable reduction in viable counts one hour later. Rita and colleagues indicated that quercetin has the potential to markedly decrease ROS production, upregulate the transcription of the type I collagen gene, and improve mitochondrial respiration. Marcelo et al. successfully demonstrated that quercetin can prevent alveolar bone loss by inhibiting the cytokine receptor activator of nuclear factor kappa-B ligand. This is achieved through the reduction of stimulating cytokines (IL-1β, TNF-α, and IL-17) and the down-regulation of osteoclasts. Due to the multifunctional properties of quercetin, it is hypothesized that an implant surface coated with quercetin could reduce osteoclast activity and bacterial invasion, while also enhancing the healing of surrounding tissues. Alba et al. demonstrated that an implant coated with quercitrin can inhibit the expression of genes associated with osteoclasts, including the calcitonin receptor and cathepsin K, thereby reducing bone resorption. However, additional attention is required for quercetin to further validate its therapeutic efficacy in the treatment of peri-implantitis [6].

Recently, bromelain-quercetin, utilized as an adjunct in quadruple therapy for the treatment of COVID-19 patients, has demonstrated significant anti-inflammatory properties [5]. Due to the scarcity of literature assessing the combination of bromelain-quercetin for oral cavity treatment, a recent study has implemented bromelain-quercetin intervention in periodontal therapy titled “Comparative Evaluation of Bromelain-Quercetin Gel with Chlorhexidine Gel as Subgingival Local Drug Delivery Following Scaling and Root Planning in Stage I /II and Grade B Periodontitis - Randomized Control Clinical Trial” [2]. The findings of this study support the preference for combination therapy instead of scaling and root planning (SRP) to attain more significant reductions in pocket depth and enhanced attachment level gains. Furthermore, local drug delivery proves to be effective in enhancing clinical parameters even three weeks post-SRP, once the pockets have stabilized [2].

Conclusion

The future of bromelain and quercetin in periodontics is undoubtedly bright, and its versatile properties as an antioxidant, anti-inflammatory, antiviral, anticancer, and cardiovascular agent make it a beacon of hope in the quest for improved healthcare and disease management [7]

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