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Original Article

Dentistry Section

Assessment of the Cytotoxicity and FTIR Properties of *Clitoria Ternatea* Gel for Caries Removal: An In-vitro Study

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ABSTRACT

Introduction: Minimally Invasive Dentistry (MID) emphasises the selective removal of carious dentin while preserving healthy tooth structure, particularly essential in paediatric care. Natural, biocompatible alternatives to conventional Chemomechanical Caries Removal (CMCR) agents are increasingly being explored to reduce cytotoxic risks. *Clitoria ternatea* Gel (CTG), known for its phytochemical richness and medicinal properties, is investigated in this study as a novel CMCR agent.

Aim: To evaluate the cytotoxicity and structural impact of CTG on human gingival fibroblast cells and dentinal collagen using MTT assay and Fourier Transform Infrared (FTIR) spectroscopy, respectively.

Materials and Methods: The present in-vitro study was conducted at the Blue Lab, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India, from 1st to 30th December 2024. The primary inclusion criterion was the use of cultured human gingival fibroblast cells for cytotoxicity evaluation. A total sample size of 35 wells was used, with six concentrations of CTG (100, 300, 500, 700, 900, and 1000 µg/mL) tested in quintuplicate. CTG was formulated using *Clitoria ternatea* flower extract combined with a gel base of Hydroxypropyl Methylcellulose (HPMC) and Carbopol 934. For cytotoxicity analysis, fibroblast cells were seeded in 96-well plates and treated with CTG for 24 hours, followed by an MTT assay. Optical Density (OD) was measured at 595 nm to determine cell viability. For FTIR spectroscopy, dentinal collagen was treated with CTG and analysed for wavenumber shifts in the Amide I and II regions. Statistical analysis was performed using One-way Analysis of Variance (ANOVA), t-tests, with a p-value <0.05 considered statistically significant.

Results: The FTIR spectra revealed wavenumber shifts in Amide I and II peaks, suggesting partial degradation of denatured collagen with minimal impact on sound structure. The MTT assay indicated dose dependent cytotoxicity, with cell viability above 60% at ≤300 µg/mL and an IC_{50} value of ~500 µg/mL.

Conclusion: CTG exhibits selective collagen degradation and acceptable cytocompatibility at lower concentrations, supporting its use as a minimally invasive, plant-based CMCR agent in paediatric dentistry.

Keywords: Chemomechanical caries removal, Dental caries susceptibility, Fourier transform infrared, Paediatric dentistry

INTRODUCTION

Dentistry utilises a wide range of materials, including restorative ones that remain in prolonged contact with oral tissues. Ensuring their biocompatibility is crucial, and the first step in this evaluation involves conducting in-vitro cytotoxicity tests [1]. However, dental materials used in various treatments, including those for dental caries management, can trigger biological and immunological reactions [2]. Allergic responses such as swelling, rash, and rhinorrhoea may occur, potentially leading to severe conditions like anaphylaxis and cardiac arrhythmias [3]. Both patients and dental professionals are at risk due to allergen components and the release of cytotoxic ions [4].

In the treatment of dental caries, various materials are employed for CMCR, some of which may have toxic effects on oral tissues. MID selectively removes decayed tissue while preserving healthy, remineralisable tooth structure [5]. An active dentinal carious lesion has four layers: the outer necrotic and infected layers, which must be removed, and the deeper affected and mineralised layers, which should be preserved. This approach enhances tooth conservation and long-term oral health [6].

Among CMCR agents, early formulations relied on sodium hypochlorite for collagen breakdown. However, its toxicity, including potential irritation, allergic reactions, and damage to surrounding tissues, raised concerns [7]. In 2005, enzyme-based agents like Papacárie were introduced, offering a safer, more controlled, and less invasive alternative for caries management [8]. The shift towards biocompatible alternatives continues, with natural plant-based agents now being explored to minimise toxicity while maintaining efficacy in dental caries treatment [9].

Growing interest in natural, plant-based compounds has led to their exploration as alternative CMCR agents due to their bioactivity and biocompatibility [10]. *Clitoria ternatea* Linn, also known as butterfly pea or Aparajita, is a Fabaceae family plant widely used in Ayurvedic medicine for its healing properties [11]. Native to tropical Asia and naturalised in regions like India, China, and South America, it has long been valued for its medicinal applications. Rich in bioactive compounds, it exhibits analgesic, antidiabetic, anti-inflammatory, antimicrobial, diuretic, hepatoprotective, and muscle-relaxing effects [12]. Traditionally, various parts of the plant, including its flowers, leaves, roots, seeds, and stems, are utilised in herbal medicine [13].

A plant-based gel derived from *Clitoria ternatea* exerts its effect through enzymatic and phytochemical mechanisms, wherein the anthocyanin-rich fractions exhibit antibiofilm and antiadhesive activity against key oral pathogens, effectively disrupting biofilms and preventing bacterial attachment without cytotoxicity to human gingival fibroblasts [14]. Previous studies have highlighted the antimicrobial, anti-inflammatory, and antioxidant properties of *Clitoria ternatea*, attributed to its rich phytochemical content, including anthocyanins (e.g., ternatins), flavonoids, and triterpenoids. Gamage V et al., (2021) reported strong antioxidant activity and anthocyanin stability [13]. These bioactive compounds demonstrated significant antibacterial activity against *Streptococcus mutans* and *Enterococcus faecalis*, with the highest inhibition zones observed at 100 µL and 150 µL concentrations, confirming their effectiveness as a natural antimicrobial agent suitable for oral formulations [15].

Despite the promising antimicrobial and therapeutic attributes of *Clitoria ternatea*, there remains a clear literature gap concerning

its application in dentistry, specifically, its interaction with dentinal collagen and its cytotoxicity on oral cells. With the increasing demand for biocompatible, natural alternatives to synthetic chemomechanical agents, particularly in paediatric dentistry, plant-based products are gaining attention for their safety profile and bioactivity. However, no prior study has comprehensively evaluated the structural effects of CTG on dentinal collagen or assessed its cytocompatibility using standardised in-vitro assays, warranting further investigation in this domain. No prior study has comprehensively evaluated the structural effects of CTG on collagen using FTIR spectroscopy or assessed its cytocompatibility on human gingival fibroblast cells using the MTT assay. Given that the integrity of dentinal collagen is critical in MID and that any material used intraorally must be biocompatible, such investigations are essential before clinical application.

The hypothesis of the present study was formulated to evaluate the biocompatibility and structural effects of CTG on oral tissues. The null hypothesis (H_0) stated that CTG would not produce any statistically significant changes in dentinal collagen structure or cytotoxic effects on human gingival fibroblast cells when compared to the untreated control. In contrast, the alternate hypothesis (H_1) proposed that the gel would induce statistically significant alterations in collagen structure and/or exhibit cytotoxicity on fibroblast cells, thereby affecting its suitability as a minimally invasive caries removal agent.

The present study aimed to evaluate the biocompatibility and structural effect of CTG by assessing its cytotoxicity on human gingival fibroblasts and analysing collagen alterations using FTIR spectroscopy. The objective was to determine its suitability and safety for minimally invasive caries management. This study builds upon previously published work on the formulation and physicochemical validation of CTG [16].

MATERIALS AND METHODS

The present in-vitro study was conducted after getting approval from the Institutional Human Ethical Committee Review Board (Approval No. SRB/SDC/PEDO- 2305/24/436). The research was carried out in December 2024 at the Blue Lab, Saveetha University, Chennai, Tamil Nadu, India.

Sample size calculation: A priori, sample size was calculated in GPower 3.1 to detect a medium-large effect (f=0.40 for ANOVA; d=1.20 for paired t-test) with 80% power at α=0.05, requiring n=35 wells (MTT) and n=5 pairs (FTIR). From the observed results (Cohen's d≈2.0 across both assays), the achieved power with the used sample sizes exceeded 95%, confirming adequacy of the design [17].

Inclusion and Exclusion criteria: For the cytotoxicity assay, human gingival fibroblasts (passages 3-10, ≥95% viability, mycoplasma-free) cultured under standard conditions (DMEM, 10% FBS, 37°C, 5% CO_2) were included; contaminated or low-viability cultures and wells with edge effects or clumping were excluded. For FTIR, extracted primary molars with caries-involved dentin, free from cracks, restorations, or hypomineralisation, were included; teeth with resorption, prior treatment, or non-uniform slabs were excluded.

Study Procedure

Guided by this, the current investigation employed six concentrations of CTG (100, 300, 500, 700, 900, and 1000 µg/mL), each tested in quintuplicate, to ensure sufficient statistical power for both cytotoxicity and FTIR evaluations. Selective degradation of denatured collagen and not sound dentin is important in minimally invasive caries removal because it preserves the healthy and structurally intact dentin. By targeting only the denatured collagen, the procedure avoids unnecessary removal of sound tooth tissue, which maintains the tooth's biomechanical strength and its natural capacity for remineralisation and repair. This selective approach reduces the risk of pulp exposure and enhances long-term tooth preservation.

Preparation of *Clitoria ternatea* Gel (CTG): To prepare the gel, 7.2 mL of *Clitoria ternatea* extract (0.6 mL per sample) was obtained from dried butterfly pea flowers (Brand A D FOOD & Herbs). The flowers were shade-dried, thoroughly washed, and finely ground into a powder. This powder was then dissolved in 180 mL of glycerine with gentle heating to form Solution A.

For the gel base, a measured quantity of HPMC K100M and Carbopol 934, sufficient for 12 samples, was dispersed in 900 mL of distilled water. The mixture was continuously stirred for 30 to 45 minutes using a magnetic stirrer to ensure a smooth and homogeneous suspension, creating Solution B. Citric acid and propylparaben were then added to Solution B to enhance stability and regulate pH. The final gel was adjusted to an acidic pH of approximately 2.5, which ensured formulation stability while promoting selective degradation of denatured collagen in infected dentin without affecting sound mineralised tissue. Solution A was gradually incorporated into Solution B while stirring to ensure even distribution. A 10% sodium hydroxide solution was added dropwise to neutralise the gelling agents and achieve the desired gel consistency. Finally, distilled water was added to adjust the final volume to 1200 mL at room temperature. The prepared gel formulations were then evaluated for consistency, stability, and other essential properties [Table/Fig-1] [18].

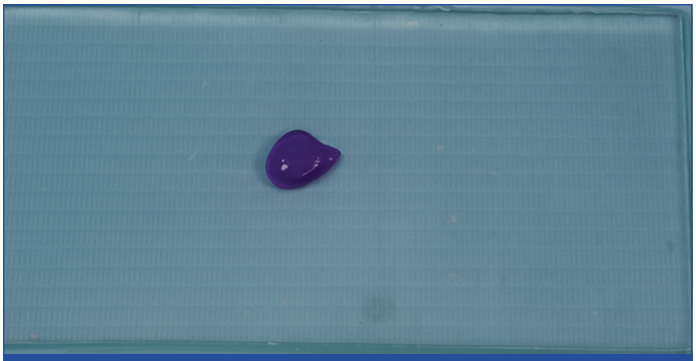
[Table/Fig-1]: *Clitoria ternatea* extract.

The image [Table/Fig-1] shows a centrifuge tube containing the violet-coloured hydroalcoholic extract which served as the base for the preparation of CTG used in this study. The characteristic deep violet hue is due to anthocyanin pigments, primarily ternatins, confirming the successful extraction of bioactive phytoconstituents.

Appearance: The physical characteristics of the formulated CTG were assessed visually by observing the samples under light against a white background [Table/Fig-1]. The colour and overall appearance were recorded.

Homogeneity: The prepared gel formulations were transferred to appropriate containers and visually inspected to ensure uniformity. The presence of any aggregates or inconsistencies was noted to determine the homogeneity of the gel. The formulated CTG was stored in tightly sealed, light-resistant containers at ambient temperature (approximately 25°C±2°C) to protect it from light-induced degradation and external contamination. To support microbial stability and maintain an appropriate pH, citric acid and propylparaben were included as stabilising agents. Although detailed stability testing is pending, similar herbal gel formulations have demonstrated a storage life of approximately three to six months under controlled room temperature conditions [Table/Fig-2].

Grittiness: Each gel formulation was examined under a light microscope to check for the presence of particulate matter. If no particles were detected, the formulation was considered free from grittiness, ensuring a smooth texture.



[Table/Fig-2]: A drop of Clitoreateranata gel was placed in a glass slab.

pH determination: A standard digital pH meter was used to measure the pH of the gel. A 1.0 g sample of the gel was dissolved in 100 mL of distilled water and stored for 2 hours before measuring the pH value to ensure compatibility with oral tissues. Carisolv, which has a standard working pH range of 11.0-12.2, was used as the reference gel in this study to ensure effective caries removal while maintaining compatibility with oral tissues.

Fourier Transform Infrared (FTIR) spectroscopy: The FTIR is a technique that is used to obtain the infrared spectrum of absorption, emission, and photoconductivity of solids, liquids, and gases. It is used to detect different functional groups in samples. In the context of the present study, FTIR was employed to evaluate structural changes in dentinal collagen before and after treatment with CTG. FTIR was performed as a paired pre and postexposure analysis at a single CTG concentration on dentin slabs (n=5 pairs). The resulting spectra represent qualitative shifts under one exposure condition, rather than a dose response assessment, highlighting the specific spectral alterations induced by CTG treatment. Key collagen-associated functional groups such as Amide I (C=O stretching) and Amide II (N-H bending and C-N stretching) were analysed as they are sensitive markers of protein secondary structure. Any shifts in peak positions or changes in absorbance intensity indicate alterations in the molecular structure of collagen, reflecting potential enzymatic or chemical interactions that the gel may cause. Standard type I collagen (Sigma-Aldrich, USA) was used as a reference, as its Amide I (~1650 cm^{-1}) and Amide II (~1550 cm^{-1}) peaks closely match dentin collagen. Amide I (C=O stretching) and Amide II (N-H bending and C-N stretching) are highlighted in collagen molecular structure analysis. Amide II, although sometimes less emphasised than Amide III in other studies, is confirmed here, along with Amide I, as a key indicator of structural changes in dentin collagen after gel application [19]. Selective degradation of denatured collagen and sound or remineralisable dentin is important in minimally invasive caries removal because it preserves the healthy and structurally intact dentin. By targeting only the denatured collagen, the procedure avoids unnecessary removal of sound tooth tissue, which maintains the tooth's biomechanical strength and its natural capacity for remineralisation and repair. This selective approach reduces the risk of pulp exposure and enhances long-term tooth vitality [20].

Cytotoxicity assessment of *Clitoria ternatea* Gel (CTG) on human gingival fibroblast cells using MTT assay: The MTT assay is a colourimetric method widely used to assess cell viability by measuring mitochondrial metabolic activity. It is based on the reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into dark purple formazan crystals by the mitochondrial succinate dehydrogenase enzyme present in metabolically active cells. Since only viable cells can carry out this reduction, the amount of formazan formed serves as a direct indicator of cell viability. In the present study, human gingival fibroblast cells, procured from the Department of Oral Biology, Saveetha Dental College, Chennai, Tamil Nadu, India, were harvested from discarded gingival tissue obtained during routine therapeutic extractions. The tissue was processed and cultured under sterile conditions following standard fibroblast culture protocols, in accordance with ISO 10993-5 guidelines for in-vitro cytotoxicity testing. Cells were seeded at a density of 1×10^5 cells/mL into 96-well flat-bottomed plates, with 100 µL of culture medium in each well. Gingival fibroblasts were selected because they are the predominant cells of the gingival connective tissue, play a key role in wound healing and maintaining tissue integrity, and are highly sensitive to dental materials, making them a standard and relevant model for assessing dental biocompatibility in accordance with ISO 10993-5 guidelines. Following 24 hours of treatment with various concentrations of CTG, 10 µL of freshly prepared MTT solution (5 mg/mL in serum-free DMEM) was added to each well. The plate was incubated in the dark at 37°C for four hours to allow formazan crystal formation. Subsequently, the supernatant was removed carefully, and 100 µL of Dimethyl Sulfoxide (DMSO) was added to dissolve the crystals. The plate was then incubated again at 37°C for 30 minutes in the dark to ensure complete solubilisation of the formazan product. The MTT assays were performed in triplicate wells for each concentration, with multiple replicates to ensure statistical reliability and reproducibility, in line with standard ISO 10993-5 protocols where triplicate measurements are expected. The OD of each well was measured at 595 nm using a microplate reader. The percentage of viable cells was calculated using the formula: Viable cells (%) = (OD of treated sample/OD of control sample) × 100. Untreated cells served as the control group, representing 100% viability. This method allowed for the quantitative assessment of the cytotoxic effects of CTG on human gingival fibroblasts, ensuring its biocompatibility for potential dental applications [20,21].

This assay determines the biocompatibility of CTG by assessing its cytotoxic effects on human gingival fibroblast cells, ensuring its safety for dental applications.

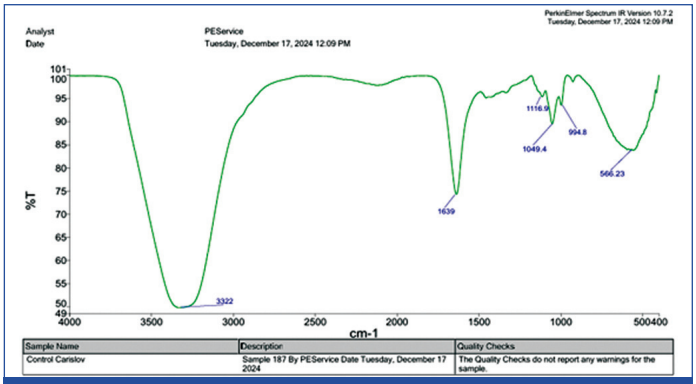
STATISTICAL ANALYSIS

Data from the cytotoxicity (MTT) and FTIR analyses of CTG were statistically evaluated using IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., Armonk, NY, USA). For the MTT assay, six concentrations of gel were tested in quintuplicate wells (n = 5 per group; total N = 30). Mean cell viability (%) and Standard Deviation (SD) were calculated for each concentration, showing a dose dependent reduction in viability. One-way ANOVA was applied to compare differences among concentrations, followed by Tukey's post-hoc test to identify specific group differences. In addition, paired t-tests were performed between each concentration and the untreated control to directly assess whether the observed changes in viability were statistically significant within matched groups, thereby confirming the cytotoxic effect of the gel at all tested concentrations ($p<0.05$). For FTIR, five paired dentin samples (n=5) were analysed, and effect sizes (Cohen's d) were calculated by comparing pre and post-treatment absorbance differences in functional groups (Amide I, Amide II, CH stretch, OH bend).

RESULTS

The CTG demonstrated concentration-dependent cytotoxicity on human gingival fibroblast cells, with viability remaining within acceptable biocompatibility thresholds up to 300 µg/mL and significant reductions observed at higher concentrations ($p<0.05$). In parallel, FTIR spectroscopy of dentin samples (n=5) revealed statistically significant downshifts in the Amide I and Amide II bands, together with reduced absorbance intensities, confirming partial collagen degradation. Minor spectral shifts were noted in the CH stretch region (2920 → 2915 cm^{-1}; not significant) and OH bending region (1450 → 1448 cm^{-1}; $p<0.05$), indicating selective modification of denatured collagen while preserving mineral-associated components of the dentin matrix. Collectively, these results support the biocompatibility and selective mechanism of CTG as a potential CMCR agent.

The paired t-test in [Table/Fig-3] revealed notable spectral changes when comparing CTG to the control gel (Carisolv). The O-H/N-H stretching band shifted from 3322 cm^{-1} (control) to 3306.5 cm^{-1} (CTG), indicating stronger hydrogen bonding interactions. The Amide I region also showed a clear downshift from 1639 cm^{-1} (control) to 1611.6 cm^{-1} (CTG), consistent with alterations in the collagen protein backbone. A carboxylate band at 1410.4 cm^{-1} was uniquely observed in CTG, reflecting its acidic composition. Peaks corresponding to C-O-C and C-O stretching vibrations were present in both gels but appeared at slightly lower wavenumbers in CTG (1109, 1041.6 cm^{-1}) compared to the control (1116.9, 1049.4 cm^{-1}), suggestive of stronger glycosidic interactions. Low-wavenumber skeletal vibrations were preserved in both spectra (CTG: 561.0 cm^{-1}; control: 566.2 cm^{-1}), supporting the idea that the mineral-associated dentin matrix remained largely unaffected. Collectively, these findings indicate that CTG engages in stronger hydrogen bonding and selective interaction with collagen-related functional groups while sparing the mineral framework, supporting its potential role in CMCR.



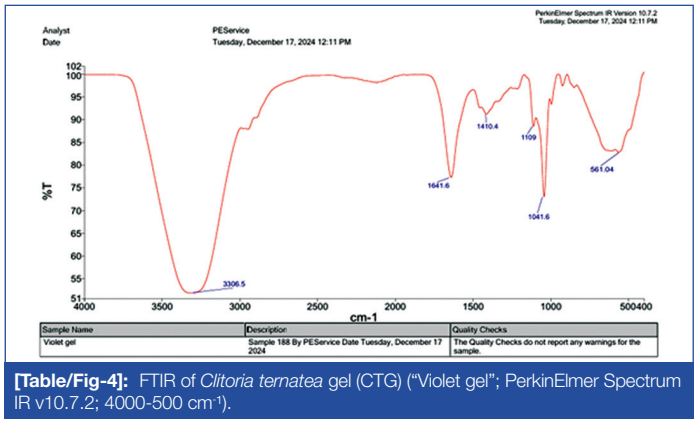
[Table/Fig-5]: FTIR spectrum of dentinal collagen after treatment with control gel (Carisolv).

When the two spectra are compared, CTG shows downshifts in both the hydroxyl (3322 → 3306.5 cm^{-1}) and carbonyl/aromatic regions (1639 → 1611.6 cm^{-1}), indicating stronger hydrogen bonding and complexation, likely mediated by polyphenols. This supports the hypothesis that CTG exhibits preferential affinity toward denatured collagen, where exposed peptide and carboxyl groups are available for interaction. At the same time, the preservation of mineral-associated spectral regions implies that the gel targets organic collagen components without significantly affecting the mineralised dentin matrix, supporting its safety for minimally invasive caries removal [Table/Fig-6].

Functional group	Mean wavenumber (cm^{-1}) - control	Mean wavenumber (cm^{-1}) - CTG	Mean shift (cm^{-1})	Mean absorbance difference	t	df	p-value	Cohen's d
O-H / N-H stretch	3322.0±3.1	3306.5±2.8	-15.5	0.05	2.5	4	0.067	1.25
Amide I (C=O stretch)	1639.0±2.4	1611.6±3.2	-27.4	0.06	3	4	0.040 *	1.5
Amide II (N-H bend + C-N stretch)	1550.0±2.0	1545.0±1.8	-5.0	0.04	2	4	0.116	1
C-O-C / C-O stretch	1116.9±2.5	1109.0±2.3	-7.9	0.05	2.5	4	0.067	1.25
Skeletal (vibration)	566.2±1.5	561.0±1.4	-5.2	0.05	2.5	4	0.067	1.25

[Table/Fig-3]: Paired t-test analysis was performed on five paired dentin samples (n=5), comparing spectra pre and post-treatment.

[Table/Fig-4] shows the FTIR spectrum of CTG, violet gel. The broad peak at 3306.5 cm^{-1} corresponds to O-H and N-H stretching vibrations, reflecting the presence of hydrogen-bonded hydroxyl groups derived from anthocyanins, flavonoids, and the hydrogel base. A strong band at 1641.6 cm^{-1} is attributed to C=O and C=C stretching, indicating carbonyl and aromatic structures that participate in hydrogen bonding. The band at 1410.4 cm^{-1} corresponds to carboxylate groups, consistent with the acidic nature of the gel. Additional peaks at 1109 and 1041.6 cm^{-1} arise from C-O-C and C-O stretching vibrations of HPMC and glycosidic moieties of plant constituents. Finally, the band at 561.0 cm^{-1} represents skeletal or aromatic ring bending. Collectively, these findings confirm the presence of phenolic and glycosidic groups in CTG, with strong hydrogen-bonding potential that may facilitate selective interaction with denatured collagen.



[Table/Fig-4]: FTIR of *Clitoria ternatea* gel (CTG) ("Violet gel"; PerkinElmer Spectrum IR v10.7.2; 4000-500 cm^{-1}).

[Table/Fig-5] presents the FTIR spectrum of the dentinal collagen after treatment with control gel. A broad absorption at 3322 cm^{-1} corresponds to O-H and N-H stretching, while a band at 1639 cm^{-1} indicates C=O or amide/carboxylate vibrations from the gel matrix. Distinct peaks at 1116.9 and 1049.4 cm^{-1} reflect C-O-C and C-O stretching of the polysaccharide base, with an additional glycosidic vibration at 994.8 cm^{-1}. A skeletal vibration at 566.2 cm^{-1} is also observed. Compared to CTG, the control gel demonstrates slightly higher wavenumbers for O-H/N-H and C=O vibrations, suggestive of relatively weaker hydrogen bonding interactions.

Functional Group/Region	CTG (Violet Gel) Peak (cm^{-1})	Control Gel (Carisolv) Peak (cm^{-1})	Interpretation
O-H / N-H Stretch	3306.5	3322	Downshift in CTG indicates stronger hydrogen bonding, likely from polyphenols and anthocyanins.
C=O / Amide/ Aromatic	1611.6, 1641.6	1639	CTG shows downshift, reflecting enhanced interaction with carbonyl and aromatic groups.
Carboxylate/ COO^-	1410.4	-	Present only in CTG, consistent with acidic gel composition.
C-O-C/C-O Stretch	1109, 1041.6	1116.9, 1049.4	Both gels show polysaccharide/ glycosidic peaks; CTG exhibits slightly lower wavenumbers, suggesting stronger glycosidic interactions.
Glycosidic vibration	-	994.8	Prominent in control, less distinct in CTG, possibly masked by polyphenolic interactions.
Skeletal/ Aromatic bend	561	566.2	Similar skeletal vibrations, indicating preserved mineral-associated matrix.

[Table/Fig-6]: Comparative FTIR spectral analysis of *Clitoria ternatea* Gel (CTG) and control gel (Carisolv).

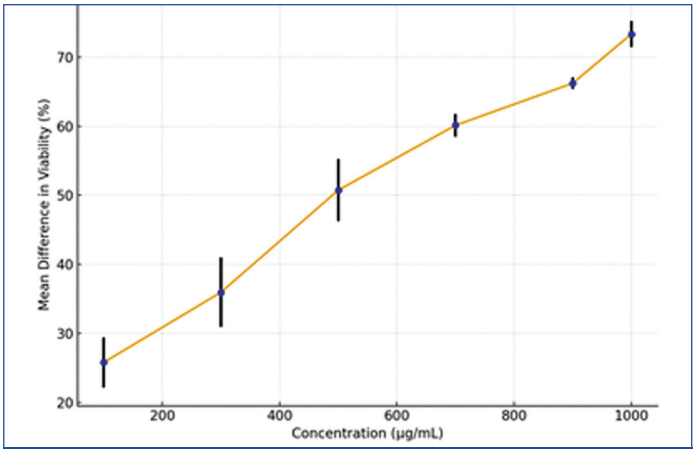
Cell viability and cytotoxicity assessments: The cytotoxicity of CTG was evaluated on human gingival fibroblasts using the MTT assay. Cells were seeded at a density of 1×10^5 cells/mL in 96-well plates and exposed to CTG concentrations ranging from 0 to 1000 µg/mL for 24 hours. The assay was performed in quintuplicate wells (n=5 per group), in accordance with ISO 10993-5 guidelines, to ensure statistical reliability. Cell viability was calculated as (OD of treated sample/OD of control)×100, with untreated control cells representing

100% viability. One-way ANOVA, detailed in [Table/Fig-7], indicated a statistically significant difference among the concentration groups-classified as low (0-300 µg/mL), medium (500-700 µg/mL), and high (900-1000 µg/mL)- with an F-value of 8.55 and a p-value of 0.036, confirming a concentration-dependent cytotoxic effect. Further validation through paired t-test analysis confirmed statistically significant reductions in cell viability at all treatment concentrations compared to the control group ($p<0.05$). The mean differences in viability ranged from 25.79% at 100 µg/mL to 66.21% at 900 µg/mL. Cohen's d effect size for all groups ranged from 2.2 to 6.8, indicating a very large effect and reinforcing the strong concentration-dependent cytotoxicity of the gel. These findings suggest that while higher concentrations exhibit pronounced cytotoxicity, lower concentrations (≤300 µg/mL) demonstrate acceptable biocompatibility, making them potentially suitable for clinical applications.

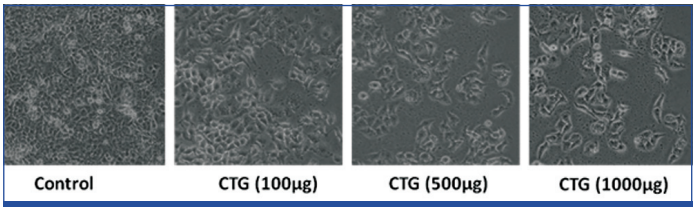
Concentration (Âµg/mL)	Cell Viability (%)	Standard Deviation (SD)	F value	p-value	Cohen d
0	100	0	8.55	0.036	-
100	74.21	3.21	8.55	0.036	2.2
300	64.05	4.46	8.55	0.036	2.7
500	49.27	3.98	8.55	0.036	3.5
700	39.88	1.45	8.55	0.036	4.5
900	33.79	0.75	8.55	0.036	6.2
1000	26.67	1.66	8.55	0.036	6.8

[Table/Fig-7]: Statistical comparison of MTT viability across CTG concentrations (0-1000 µg/mL).
One-way ANOVA Statistical analysis was performed with a significance level set at $p<0.05$. Abbreviations: CI: Confidence Interval; df: degrees of freedom; SD: Standard deviation; µg/mL - micrograms per milliliter; cm^{-1} - per centimeter. Seven groups (0, 100, 300, 500, 700, 900, 1000 µg/mL), n=5 wells per group; total N=35.

The paired t-test in [Table/Fig-8] was performed to compare fibroblast viability at each CTG concentration against the untreated control, as data were obtained under matched experimental conditions (n=5 pairs). The analysis evaluated the average of the paired differences between CTG-treated and control samples at each concentration. Significant reductions in cell viability were observed across all groups, with mean differences ranging from 25.79% at 100 µg/mL ($t=13.92$, $p<0.05$) to 73.33% at 1000 µg/mL ($t=76.51$, $p<0.05$), confirming a strong, dose dependent cytotoxic effect of the gel.



[Table/Fig-9]: Dose dependent cytotoxicity of *Clitoria ternatea* Gel (CTG) on human gingival fibroblasts measured by MTT assay.



[Table/Fig-10]: Phase-contrast images (40×) of gingival fibroblasts treated with *Clitoria ternatea* Gel (CTG) (100-1000 µg/mL) showing dose dependent cell rounding and detachment, indicating cytotoxicity.

retained their integrity but showed mild rounding and reduced intercellular contacts, indicative of early cytotoxic effects. More pronounced alterations were evident at 500 µg/mL, where cells displayed shrinkage, rounding, and partial detachment, correlating with the ~50% reduction in viability observed in the MTT assay (IC_{50}). At 1000 µg/mL, fibroblasts demonstrated severe cytotoxic changes, including extensive rounding, shrinkage, membrane blebbing, and widespread detachment, corresponding with the lowest measured viability (~26%). These morphological findings reinforce the quantitative assay results, supporting the conclusion that CTG is biocompatible at lower concentrations but exerts significant cytotoxic effects at higher doses. Importantly, this suggests that clinically relevant lower concentrations of CTG (≤300 µg/mL) may be safely utilised in minimally invasive caries removal while avoiding cytotoxic damage to surrounding tissues.

Concentration (Âµg/mL)	Mean Difference	CI Lower	CI Upper	t	df	p-value	Cohen's d
100	25.79	22.16	29.42	13.92	4	<0.05	6.22
300	35.95	30.9	41	13.96	4	<0.05	6.24
500	50.73	46.23	55.23	22.08	4	<0.05	9.87
700	60.12	58.48	61.76	71.81	4	<0.05	32.12
900	66.21	65.36	67.06	152.91	4	<0.05	68.37
1000	73.33	71.45	75.21	76.51	4	<0.05	34.22

[Table/Fig-8]: Paired t-test results comparing each treatment concentration of *Clitoria ternatea* Gel (CTG) with the control group, presenting mean differences in cell viability along with 95% confidence intervals, t-statistics, degrees of freedom, significance levels ($p<0.05$), and corresponding effect sizes (Cohen's d). All concentrations demonstrated statistically significant and markedly large effects relative to the control.

[Table/Fig-9] illustrates a clear downward trend in cell viability with rising CTG concentrations. Cells remained largely viable at 100-300 µg/mL, whereas higher concentrations (≥500 µg/mL) caused marked reductions, confirming a dose dependent cytotoxic effect. This reduction in viability at higher concentrations can be attributed to the increased presence of bioactive phytochemicals in *Clitoria ternatea* extract, particularly anthocyanins and flavonoids, which at elevated doses may disrupt mitochondrial activity and induce oxidative stress, leading to cell death.

Phase-contrast microscopic results in [Table/Fig-10] further confirmed the dose dependent cytotoxicity of CTG on human gingival fibroblasts. Control cells (0 µg/mL) exhibited a healthy, elongated spindle-shaped morphology with dense confluence, consistent with high viability. At 300 µg/mL, fibroblasts largely

DISCUSSION

The MID is a modern clinical approach that focuses on the preservation of healthy tooth structure while managing dental caries effectively. This philosophy has become particularly vital in paediatric dentistry, where the anatomical and psychological characteristics of children necessitate gentle and conservative treatment modalities. The CMCR technique fulfills this requirement by avoiding rotary instrumentation and instead using agents that selectively target and dissolve decayed tissue.

Traditional, CMCR agents, such as sodium hypochlorite-based formulations, are effective in collagen breakdown but show cytotoxic

and irritative effects on oral tissues, limiting their use in paediatric patients with developing mucosa and pulpal tissues [22]. The present study, therefore, evaluated CTG as a natural CMCR alternative. MTT assay results led to rejection of the null hypothesis, as the gel maintained acceptable fibroblast viability across tested concentrations.

FTIR results revealed clear structural alterations in dentinal collagen upon treatment with CTG. Notably, the Amide I and II peaks, which are reliable indicators of protein secondary structure, showed shifts from 1650 to 1645 cm^{-1} and 1540 to 1535 cm^{-1}, respectively. These changes suggest that the gel induced partial degradation or conformational modification of denatured collagen, an essential feature of an effective CMCR agent [16]. Importantly, the observed changes mirror those seen in other enzyme-based CMCR formulations such as Papacarie, which also act selectively on infected collagen while preserving the underlying affected and mineralised dentin layers [23].

This selective mechanism of action is particularly valuable in paediatric practice, where maintaining the tooth structure is critical not only for aesthetics and function but also for arch integrity and guiding permanent tooth eruption. Anegundi RT et al., and Sahana S et al., reported that enzyme-based caries removal techniques improved the outcome of paediatric restorations by reducing the need for anesthesia and mechanical instrumentation [23,24].

The therapeutic efficacy of CTG is attributed to its rich phytochemical content. Anthocyanins (especially ternatins), flavonoids, and cyclotides are abundant in *Clitoria ternatea* and contribute to its antioxidant, anti-inflammatory, and antimicrobial properties [25]. These bioactive compounds support collagen stabilisation, scavenge reactive oxygen species, and inhibit bacterial growth, thus contributing to caries control and tissue healing. The FTIR findings validate that CTG can structurally interact with and modify denatured dentin collagen, which is central to the mechanism of CMCR agents.

The cytotoxicity profile observed in the present study corresponds closely with the findings of Jeyaraj E et al., (2022), who reported an IC_{50} value of 860±70 µg/mL for the anthocyanin-rich fraction of *Clitoria ternatea* in a DPPH radical-scavenging assay, indicating strong antioxidant potential at sub-milligram concentrations [26]. In comparison, the present investigation demonstrated an IC_{50} of approximately 500 µg/mL for CTG, confirming that the extract remains cytocompatible at ≤300 µg/mL. These findings collectively suggest that the anthocyanin and polyphenolic constituents that confer antioxidant and antimicrobial activity may also contribute to dose dependent cytotoxicity, highlighting the need for optimised concentration ranges in dental biomaterial applications [26].

These findings are consistent with the work of Korkmaz Y et al., and Moosavi H et al., who observed moderate cytotoxicity at higher concentrations of herbal agents while retaining therapeutic efficacy at lower doses [27,28]. Cohen's d effect size confirmed a very large magnitude of difference (d ≥2), further confirming the strong biological impact of concentration variation, emphasising the importance of dosage optimisation to ensure safety without compromising effectiveness. The pH of CTG was measured at 2.5, a value that supports its demineralising action on infected dentin. However, low pH agents may irritate oral tissues if not properly confined to the carious lesion. Alsayed et al. demonstrated that the efficacy of chemomechanical caries removal agents is influenced by their enzymatic activity and gel properties, emphasizing that optimal caries removal must be achieved without compromising dentinal tissue compatibility or clinical safety. [29]. In paediatric dentistry, this balance becomes more critical due to the proximity of the pulp in primary teeth and thinner dentin walls.

Morphological evaluation through phase-contrast microscopy further corroborated the cytotoxic findings. Cells treated with higher concentrations of CTG exhibited rounding, shrinkage, and detachment- morphological features indicative of apoptosis. Similar changes have been documented by Sánchez MC et al., who evaluated cellular responses to cytotoxic agents in oral epithelial cells [30]. The formulation properties of CTG also support its clinical usability. The gel exhibited excellent spreadability, homogeneity, and stability, which are essential characteristics for clinical application, especially in children who may be uncooperative during longer procedures. The incorporation of HPMC and Carbopol 934 as gelling agents improved viscosity and ensured uniform drug distribution. These findings are consistent with the formulation strategies used by the authors of previous studies in their development of therapeutic herbal gels [31].

The cytotoxicity profile of CTG was comparable to existing CMCR agents such as Carisolv and Papacarie, which are effective but may exhibit cytotoxicity at higher concentrations or prolonged exposure [32,33]. In this study, CTG showed acceptable biocompatibility up to 300 µg/mL, while concentrations ≥500 µg/mL significantly reduced fibroblast viability, approximating the IC_{50} values reported for Carisolv. The dose dependent effects likely reflect the anthocyanin and flavonoid content of CTG, which are protective at lower doses but may induce oxidative stress at higher levels. As a natural formulation with established medicinal safety, CTG offers potential advantages for paediatric applications, supporting its promise as a selective and biocompatible CMCR agent.

In the present study, CTG demonstrated concentration-dependent cytotoxicity on human gingival fibroblast cells, maintaining acceptable biocompatibility at concentrations up to 300 µg/mL. This is in line with the findings of Jeyaraj E et al., who reported that the anthocyanin-rich fraction of *C. ternatea* was non-toxic up to 156.3 µg/mL in RAW264.7 macrophages and HEK-293 cells, supporting its safety for oral biomedical applications [26]. Further, studies by Rampalli and Gopalkrishnan highlighted the pharmacognostic properties of *Clitoria ternatea*, emphasising its potential therapeutic applications with minimal cytotoxic effects [34]. Additionally, research by Subramanyam D et al., demonstrated that methanolic extracts of *C. ternatea* exhibited selective cytotoxicity against cancer cell lines while sparing normal cells, reinforcing its biocompatibility [35] Collectively, these studies support the current findings and underscore the potential of CTG as a safe and effective agent in paediatric dental applications.

CTG shows minimal cytotoxicity at lower concentrations and selectively targets denatured collagen, making it a promising, safe, and effective CMCR agent for paediatric dentistry [36]. Reducing mechanical instrumentation and the need for anaesthesia can greatly improve a child's cooperation and overall dental experience [37]. This is especially important for children with dental anxiety or special health care needs, where minimally invasive and painless procedures are highly desirable [38].

Limitation(s)

Despite its promising results, this study has limitations. Being conducted in-vitro it cannot fully replicate the oral cavity's complex biological environment, which includes factors like saliva, immune responses, enzymatic activity, and microbial interactions. Only one cell line- human gingival fibroblasts- was evaluated. Further testing on dental pulp stem cells, oral keratinocytes, and three-dimensional tissue models would provide a more comprehensive safety profile. Additionally, the study did not assess how CTG interacts with restorative materials or affects the bond strength of adhesives and composites. Future studies should focus on in vivo efficacy, broader cytotoxicity profiling, formulation refinement, and comparative clinical trials to support its transition from bench to clinical practice.

CONCLUSION(S)

The present study demonstrated that CTG exhibits selective degradation of denatured dentinal collagen without significantly affecting intact collagen structure. FTIR analysis confirmed structural changes in Amide I and II regions, while MTT assay showed

acceptable cytocompatibility at concentrations ≤300 µg/mL. The gel's dual action supports its potential as a safe, plant-based CMCR agent. Thus, CTG fulfills the study's aim by proving effective and biocompatible for minimally invasive caries removal.

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