

## Original Article

# Cytotoxic effects of clear aligner thermoplastic materials on human gingival fibroblasts: Influence of material and thickness

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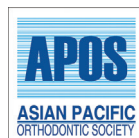
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## ABSTRACT

**Objectives:** Clear aligner therapy has gained widespread acceptance as an esthetic orthodontic option, but concerns remain regarding the biological safety of thermoplastic materials in prolonged intraoral use. This study evaluated the *in vitro* cytotoxic effects of six commercially available clear aligner thermoplastic materials, Erkodur, Duran+, Imprelon, CA Pro+, Monoflex, and Leone, at different thicknesses (0.5, 0.6, 0.75, and 1.0 mm) and under varying thermoforming conditions, on human gingival fibroblasts (HGF)

**Material and Methods:** Primary HGFs were cultured and exposed to eluents from thermoformed aligner samples prepared under three clinical simulation scenarios: As received, crowding, and spacing. Standardized samples were sterilized using ultraviolet light and stored in artificial saliva at 37°C to replicate the oral environment. The eluents were collected after 7, 14, and 21 days and diluted to 20% volume/volume percentage for testing. Cytotoxicity was evaluated through the implementation of the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, with cell viability being quantified through the utilization of spectrophotometric analysis. Statistical analysis was performed using Friedman's test and Mann-Whitney U-test, with statistical significance set at  $P < 0.05$ .

**Results:** Cell viability varied according to material type, thickness, and incubation period. Overall, cell viability declined significantly from day 7 (T0) to 21 (T2) across all groups. Among the 0.5 mm samples (pre-thermoformed sheet, crowding and spacing), CA Pro+ demonstrated the highest viability, Erkodur was intermediate, and Duran+ was lowest. The Duran+ sheet exhibited superiority over the Erkodur sheet in terms of pre-thermoformed sheets and crowding, whereas the Erkodur sheet demonstrated superiority over the Duran+ sheet in terms of spacing. In the case of 0.75-mm sheets, Leone exhibited the highest values under as-received conditions. Conversely, under conditions of crowding and spacing, Imprelon demonstrated the highest values, yielding the top values at 7/14/21 days among 0.75-mm materials (85.48%, 84.11%, 81.78%, respectively). Across all materials, there was a decline in viability over time (T0>T1>T2; all  $P = 0.007$ ), yet the majority of values remained within the slight toxicity range.

**Conclusion:** All tested thermoplastic clear aligner materials demonstrated acceptable biocompatibility, with only slight reductions in viability. The performance of the polyethylene terephthalate glycol-based (CA Pro+, Duran+, Erkodur) and polyurethane-based (Imprelon) materials was found to be optimal, particularly at greater thicknesses. These findings supported their safe clinical use while emphasizing the importance of careful material and thickness selection for optimal outcomes.

**Keywords:** Clear aligners, Cytotoxicity, Human gingival fibroblasts, Thermoplastic materials

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## INTRODUCTION

Clear aligner therapy (CAT) has become an increasingly popular alternative to fixed orthodontic appliances, primarily due to its esthetic appeal, patient comfort, and ease of maintaining oral hygiene.<sup>[1,2]</sup> Unlike conventional braces, aligners are removable thermoplastic trays designed to deliver controlled forces for gradual tooth movement.<sup>[3]</sup> Their versatility has expanded their use not only for minor tooth alignment but also for increasingly complex malocclusions, broadening their role in modern orthodontics.<sup>[4]</sup>

The polymers most commonly used for aligner fabrication include polyethylene terephthalate glycol (PETG) and polyurethane-based materials, typically processed through thermoforming.<sup>[5,6]</sup> While these materials are generally regarded as safe, their prolonged intraoral exposure to fluctuating pH, salivary enzymes, and temperature variations may promote degradation and the leaching of residual monomers.<sup>[7,8]</sup> Such by-products have been implicated in cellular changes, including reduced viability, altered membrane integrity, and potential inflammatory responses in surrounding oral tissues. A recent position paper by the World Federation of Orthodontists also emphasized the importance of assessing not only the biological but also the environmental impact of polymer-based orthodontic materials.<sup>[9]</sup>

Human gingival fibroblasts (HGFs) represent a critical cell population for evaluating aligner biocompatibility because of their central role in periodontal health and their direct contact with aligner surfaces during wear.<sup>[10]</sup> Previous *in vitro* investigations have reported varying degrees of cytotoxicity associated with different aligner systems and material formulations, ranging from negligible to measurable reductions in cell viability.<sup>[11-13]</sup> The inconsistencies across studies are attributed to variations in polymer composition, thickness, thermoforming conditions, and experimental design.<sup>[5,14]</sup>

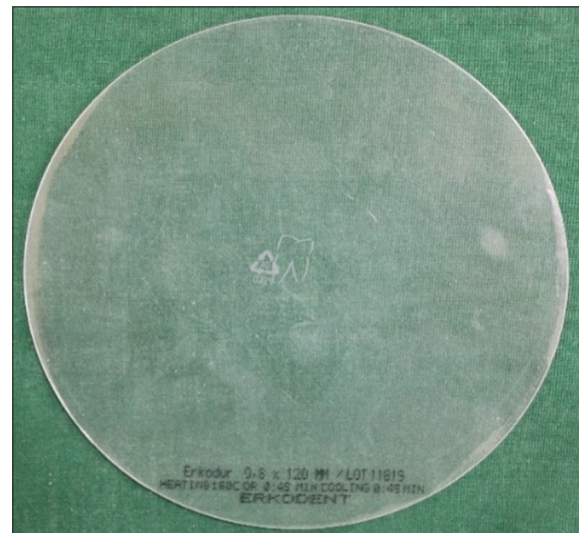
More recently, systematic reviews and *in vitro* studies have reaffirmed that while clear aligners are largely biocompatible, certain thermoplastic formulations may still induce moderate cytotoxic effects and chemical leaching, underscoring the need for ongoing evaluation.<sup>[6,7]</sup> Given the rapid adoption of clear aligners worldwide, further systematic investigations of their biological safety are warranted. The present study was therefore designed to compare, under standardized *in vitro* conditions, the cytotoxic effects of six commercially available thermoplastic materials of varying thicknesses and thermoforming scenarios on HGFs using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay.

## MATERIAL AND METHODS

### Preparation of specimens

Six commercially available thermoplastic aligner materials, Erkodur, Duran+, Imprelon, CA Pro+, Monoflex, and Leone,

were selected for testing. Each material was evaluated at four thicknesses (0.5, 0.6, 0.75, and 1.0 mm). These thickness values correspond to the nominal thickness before thermoforming as specified by the manufacturers. Thermoforming was performed using the BIOSTAR® thermoforming unit (Scheu Dental, GmbH, Iserlohn, Germany) according to the manufacturer's recommended parameters for temperature, pressure, and cooling. To simulate clinical conditions, specimens were prepared in three categories: As-received (pre-thermoformed) [Figure 1], crowding [Figure 2], and spacing [Figure 3]. After thermoforming, aligners were trimmed, edges were smoothed, and the incisal region was isolated for testing, as this area undergoes the greatest stress during orthodontic movement [Figure 4].



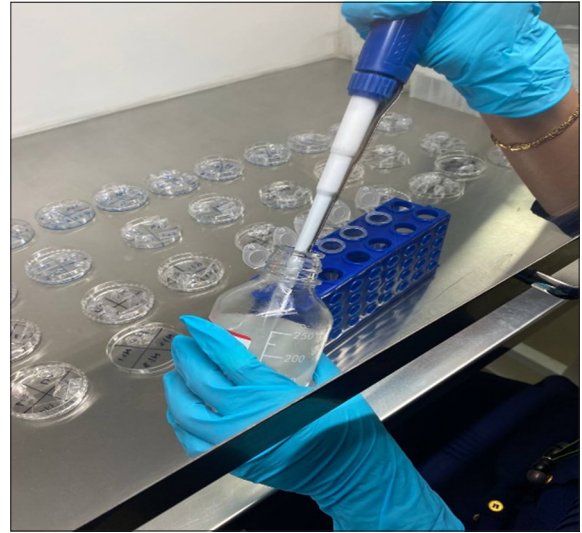
**Figure 1:** Pre-thermoformed aligner sheets before processing.



**Figure 2:** Thermoformed aligner sheets on the crowding model.



**Figure 3:** Thermoformed aligner sheets on the spacing model.



**Figure 5:** Immersion of sterilized aligner samples in artificial saliva at 37°C.



**Figure 4:** Trimming of the incisal region of thermoformed aligners for testing.

### Elution and exposure

Prepared specimens were sterilized by ultraviolet (UV) irradiation in a vertical laminar airflow chamber for 6 h. Each specimen was immersed in artificial saliva (Sigma-Aldrich, USA) and stored at 37°C to simulate intraoral conditions [Figure 5]. Eluents were collected after 7, 14, and 21 days of immersion. To prevent excessive dilution of the culture medium, eluents were standardized to 20% v/v before cytotoxicity testing.<sup>[12]</sup> HGFs were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well and incubated for 24 h, after which the culture medium was replaced with 200  $\mu$ L of eluent and incubated for another 24 h. Cells maintained in

fresh Dulbecco's Modified Eagle medium (DMEM) without eluents served as the negative control (100% viability).<sup>[8]</sup>

### Cell culture

Primary HGFs were obtained from a certified cell culture laboratory. Cryopreserved HGFs were thawed and cultured following the established protocol.<sup>[15]</sup> Cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics, and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells from passages 3 to 5 were used for all experiments.

### Cytotoxicity assay and statistical analysis

Cell viability was determined using the MTT assay (Sigma Chemical Co., Milan, Italy). After exposure to eluents, 100  $\mu$ L of MTT solution (1 mg/mL in phosphate-buffered saline) was added per well and incubated for 1 h at 37°C. The solution was then discarded, and dimethyl sulfoxide was added to dissolve the formazan crystals. Optical density (OD) was measured at 590 nm using a spectrophotometer (UV-2600, Shimadzu Corporation, Kyoto, Japan). Cell viability (%) was calculated as: Cell viability (%) = (OD of treated cells/OD of control cells)  $\times$  100.<sup>[16]</sup> All experiments were conducted in triplicate. Data were analyzed using Statistical Package for the Social Sciences software v20.0 (IBM, Chicago, USA). Since the data were not normally distributed, non-parametric tests were applied. Intragroup comparisons were performed using Friedman's test with *post hoc* Mann-Whitney U-test, while intergroup comparisons were analyzed with the Mann-Whitney U-test. A  $P < 0.05$  was considered statistically significant.

**RESULTS**

The cell viability data for all materials and conditions are summarized in [Tables 1-4]. Across the dataset, there was a decrease in viability over time (7> 14> 21 days) for every group (all  $P = 0.007$ ), with most values lying between 60% and 90%, indicating slight overall toxicity.

For 0.5 mm thickness materials [Table 1]. For the as-received, crowding, and spacing setups, the ranking was consistent: CA Pro+ showed the highest viability, Erkodur was intermediate, and Duran+ was lowest at T0, T1, and T2. The time trend

(T0> T1> T2) held for each material–scenario combination [Figure 6].

For 0.6 mm thickness materials [Table 2]. Duran+ exceeded Erkodur under as-received and crowding conditions at all-time points, with the overall peak recorded for Duran+ (crowding) at T0 = 85.6%. Under spacing, Erkodur exceeded Duran+ at T0, T1, and T2. In all 0.6 mm groups, viability declined from T0 to T2 [Figure 7].

For 0.75 mm thickness materials [Table 3]. Under as-received conditions, Leone showed the highest values at each time

**Table 1: Cell viability (%) for 0.5 mm sheets (as-received, crowding, spacing) at 7, 14, and 21 days (mean±SD).**

0.5 mm thickness material								
Group	% Cell viability (7 days) (A)		% Cell viability (14 days) (B)		% Cell viability (21 days) (C)		P-value	Post hoc
	Mean	SD	Mean	SD	Mean	SD		
Pre-thermoformed								
Erkodur	73.249	0.864	71.550	0.864	69.384	0.864	0.007	A>C
Duran+	69.170	0.864	66.994	0.864	64.827	0.864	0.007	A>C
CA pro+	81.692	0.864	79.516	0.864	77.349	0.864	0.007	A>C
Crowding								
Erkodur	75.622	0.864	73.446	0.864	71.280	0.864	0.007	A>C
Duran+	68.469	0.864	66.293	0.864	64.127	0.864	0.007	A>C
CA pro+	81.644	0.864	79.468	0.864	77.302	0.864	0.007	A>C
Spacing								
Erkodur	73.583	0.864	71.407	0.864	69.240	0.864	0.007	A>C
Duran+	64.152	0.864	61.976	0.864	59.809	0.864	0.007	A>C
CA pro+	79.908	0.864	77.732	0.864	75.565	0.864	0.007	A>C

SD: Standard deviation, P- value significant at: 0.05

**Table 2: Cell viability (%) for 0.6 mm sheets (as-received, crowding, spacing) at 7, 14, and 21 days (mean±SD).**

0.6 mm thickness material								
Group	% Cell viability (7 days) (A)		% Cell viability (14 days) (B)		% Cell viability (21 days) (C)		P-value	Post hoc
	Mean	SD	Mean	SD	Mean	SD		
Pre-thermoformed								
Erkodur	74.921	0.864	72.745	0.864	70.579	0.864	0.007	A>C
Duran+	85.229	0.864	83.053	0.864	80.886	0.864	0.007	A>C
Crowding								
Erkodur	74.906	0.864	72.729	0.864	70.563	0.864	0.007	A>C
Duran+	85.659	0.864	83.483	0.864	81.316	0.864	0.007	A>C
Spacing								
Erkodur	78.649	0.864	76.473	0.864	74.307	0.864	0.007	A>C
Duran+	72.548	0.864	70.372	0.864	68.205	0.864	0.007	A>C

SD: Standard deviation, P- value significant at: 0.05

**Table 3:** Cell viability (%) for 0.75 mm sheets (as-received, crowding, spacing) at 7, 14, and 21 days (mean±SD).

0.75 mm thickness material								
Group	% Cell viability (7 days) (A)		% Cell viability (14 days) (B)		% Cell viability (21 days) (C)		P-value	Post hoc
	Mean	SD	Mean	SD	Mean	SD		
Pre-thermoformed								
Erkodur	69.043	0.864	66.867	0.864	64.700	0.864	0.007	A>C
Duran+	71.002	0.864	68.826	0.864	66.660	0.864	0.007	A>C
Monoflex	73.854	0.864	71.678	0.864	69.511	0.864	0.007	A>C
Leone	81.565	0.864	79.389	0.864	77.222	0.864	0.007	A>C
Imprelnon	78.652	0.535	77.249	0.649	74.214	0.307	0.007	A>C
Crowding								
Erkodur	77.327	0.864	75.151	0.864	72.984	0.864	0.007	A>C
Duran+	77.518	0.864	75.342	0.864	73.175	0.864	0.007	A>C
Monoflex	74.906	0.864	72.729	0.864	70.563	0.864	0.007	A>C
Leone	78.299	0.864	76.123	0.864	73.956	0.864	0.007	A>C
Imprelnon	84.073	0.755	82.916	0.756	80.792	1.044	0.007	A>C
Spacing								
Erkodur	75.527	0.864	73.351	0.864	71.184	0.864	0.007	A>C
Duran+	75.256	0.864	73.080	0.864	70.913	0.864	0.007	A>C
Monoflex	73.727	0.864	71.550	0.864	69.384	0.864	0.007	A>C
Leone	78.825	0.864	76.648	0.864	74.482	0.864	0.007	A>C
Imprelnon	85.480	1.086	84.108	1.125	81.777	0.946	0.007	A>C

SD: Standard deviation, P- value significant at: 0.05

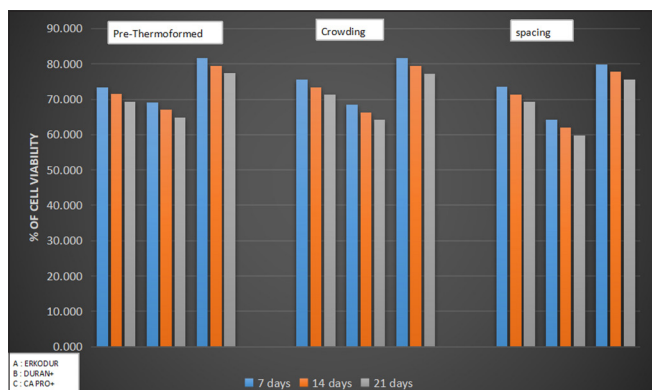
**Table 4:** Cell viability (%) for 1.0 mm sheets (as-received, crowding, spacing) at 7, 14, and 21 days (mean±SD).

1 mm thickness material								
Group	% Cell viability (7 days) (A)		% Cell viability (14 days) (B)		% Cell viability (21 days) (C)		P-value	Post hoc
	Mean	SD	Mean	SD	Mean	SD		
Pre-thermoformed								
Erkodur	75.527	0.864	73.351	0.864	71.184	0.864	0.007	A>C
Duran+	78.697	0.864	76.521	0.864	74.354	0.864	0.007	A>C
Crowding								
Erkodur	75.049	0.864	72.873	0.864	70.706	0.864	0.007	A>C
Duran+	84.193	0.864	82.017	0.864	79.851	0.864	0.007	A>C
Spacing								
Erkodur	77.709	0.864	75.533	0.864	73.367	0.864	0.007	A>C
Duran+	77.630	0.864	75.454	0.864	73.287	0.864	0.007	A>C

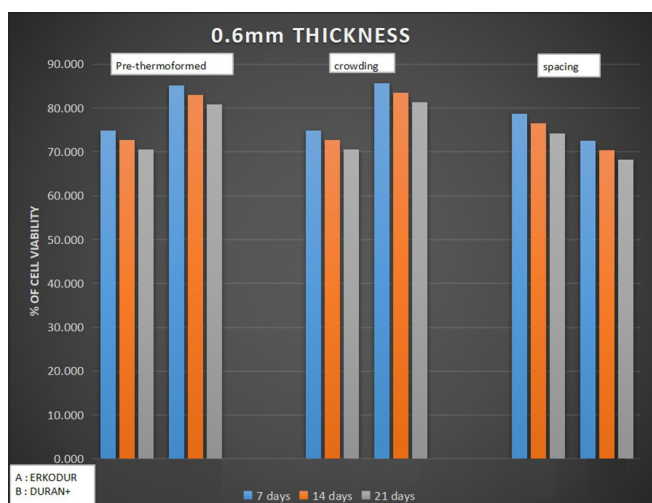
SD: Standard deviation, P- value significant at: 0.05

point. Under crowding and spacing, Imprelnon had the highest values and produced the top 0.75 mm series (spacing: T0 = 85.4%, T1 = 84.1%, T2 = 81.7%). The time-dependent decrease was present across all materials and scenarios [Figure 8].

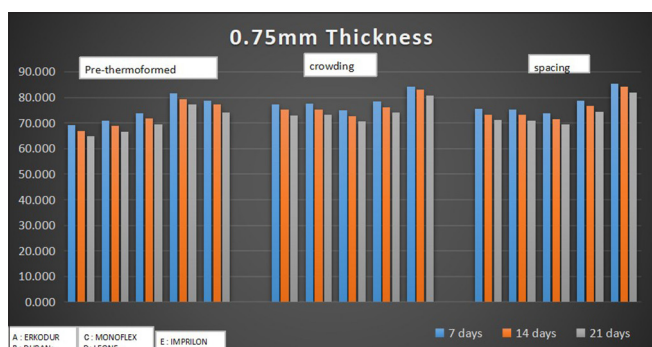
For 1 mm thickness materials [Table 4]. (Erkodur and Duran+ only.) Duran+ exceeded Erkodur in as-received and crowding conditions at all-time points, whereas Erkodur exceeded Duran+ in spacing at T0–T2. All groups again showed T0> T1> T2 [Figure 9].



**Figure 6:** Cell viability of thermoplastic aligner materials (Erkodur Duran+ and CA Pro+) at 0.5 mm thickness measured over 7, 14, and 21 days.



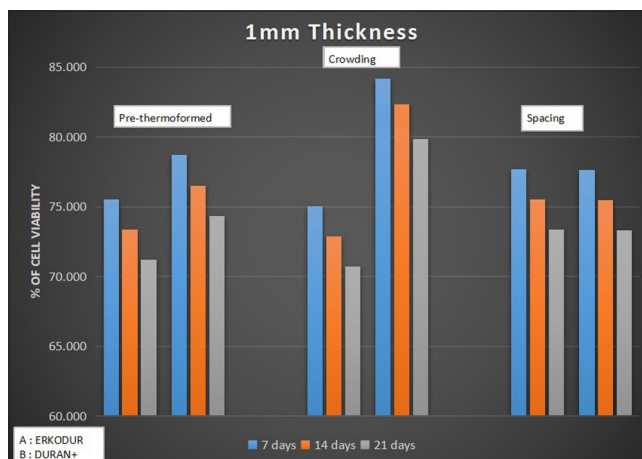
**Figure 7:** Cell viability of thermoplastic aligner materials (Erkodur and Duran+) at 0.6 mm thickness measured over 7, 14, and 21 days.



**Figure 8:** Cell viability of five thermoplastic aligner materials (Erkodur, Duran+, Monoflex, Leone and Imprelon) at 0.75 mm thickness measured over 7, 14, and 21 days.

## DISCUSSION

The popularity of CAT as a preferred orthodontic treatment option has grown rapidly, primarily due to its favourable



**Figure 9:** Cell viability of five thermoplastic aligner materials (Erkodur and Duran+) at 1 mm thickness measured over 7, 14, and 21 days.

impact on patients' aesthetics, the ease of its use, and the positive effect it has on patients' oral hygiene.<sup>[1]</sup> However, the long-term intraoral use of polymer-based materials raises concerns regarding their biological safety. Thermoplastic sheets utilised in aligners are subjected to mechanical forces, temperature fluctuations, and salivary enzymes, all of which have the potential to modify their surface properties and result in the leaching of residual monomers or degradation products.<sup>[5,17-20]</sup> The present study evaluated the cytotoxic potential of six commonly used aligner materials, tested at varying thicknesses and under different thermoforming conditions, using HGFs.

Overall, the results demonstrated that all aligner materials tested exhibited only slight cytotoxicity, with cell viability values generally between 60% and 90%. This indicates that the materials fall within the acceptable range of biocompatibility, consistent with the International Organization for Standardization 10993-5 standard that considers viability above 70% as non-cytotoxic.<sup>[21]</sup> The gradual decline in viability from day 7 to day 21 observed across all groups supports the hypothesis that prolonged exposure increases the release of substances from polymers, a finding consistent with earlier reports that aligners can release trace amounts of monomers over time, though usually not at clinically significant levels.<sup>[8,22]</sup> In a few experimental conditions, cell vitality dropped below the 70% threshold, and these findings should be interpreted with caution as they arise from a short-term static *in-vitro* model that does not fully reproduce the clearance and dilution afforded by salivary flow *in vivo*. Nevertheless, the behaviour of fixed or removable retainers that may be worn continuously for several years warrants specific investigation, because material ageing and cumulative degradation could potentially alter their long-term biocompatibility profile.

Our study also demonstrated that sheet thickness exerts a significant influence on the degree of toxicity exhibited. It was demonstrated that sheets of reduced thickness (0.5 mm) exhibited diminished viability in comparison with those measuring 0.75 mm and 1 mm thickness sheets. This may be due to greater stress concentrations during thermoforming, which can lead to increased surface degradation and higher release of breakdown products. Previous studies have also observed higher cytotoxicity with thinner thermoplastic materials, likely because thinner layers are less able to buffer or dilute the leachable component.<sup>[15]</sup> In contrast, thicker specimens demonstrated better stability, maintaining higher cell viability over time.

Thermoforming conditions also affected cytotoxicity. As-received sheets consistently showed slightly higher cell viability than thermoformed ones. This can be explained by the heat and pressure involved in thermoforming, which may induce structural changes, create microcracks, or alter surface chemistry, thereby increasing the potential for leaching.<sup>[23,24]</sup> Among thermoformed groups, crowding models demonstrated the lowest viability, likely due to greater strain and stress during adaptation to irregular tooth positions. This observation highlights that clinical conditions with more complex tooth movements may subject aligners to additional mechanical stress, potentially accelerating material degradation.

In comparing individual materials, certain brands demonstrated superior performance. For example, CA Pro+ consistently exhibited the highest viability among 0.5 mm sheets, while Imprelon performed best at 0.75 mm under crowding and spacing conditions. Leone performed well in the as-received state, but its values declined more noticeably after thermoforming. These differences can be attributed to variations in polymer composition. PETG-based materials such as Erkodur and Duran+ are known for their clarity and toughness, but may vary in residual monomer content depending on manufacturing.<sup>[25]</sup> Polyurethane-based materials such as Imprelon, on the other hand, may offer greater elasticity and resistance to stress cracking, which could explain their relatively favourable biocompatibility. Similar findings were reported in studies, where different aligner brands exhibited variable cytotoxic effects despite being marketed for the same clinical purpose.<sup>[15,8]</sup>

From a clinical perspective, the present findings are reassuring, although aligners exhibited slight toxicity *in vitro*, the majority of values remained above the 70% viability threshold, suggesting they are unlikely to pose significant biological risks under normal clinical use. Furthermore, the *in vivo* oral environment comprises dynamic salivary flow, pH buffering, and protein adsorption, which may further dilute and neutralise any released substances.<sup>[8]</sup> This suggests that the actual clinical toxicity is likely to be lower than that measured under static *in vitro* conditions.

Some limitations of the *in vitro* setting include its inability to fully replicate the complexity of the oral environment, where factors such as enzymatic degradation, food components, and thermal cycling may influence material behaviour. *In vivo*, aligners are continuously exposed to cyclic masticatory loading and abrasion, hydrolytic degradation by saliva, and repeated temperature fluctuations associated with food and beverages. These stresses may accelerate surface roughness, microcrack formation, and polymer chain reaction, potentially increasing the release of degradation products. At the same time, salivary flow, buffering capacity, and protein adsorption may partially mitigate local cytotoxic effects. Future experimental designs that incorporate simulated mastication, thermal cycling, and salivary hydrolysis would therefore provide a more realistic approximation of clinical wear conditions. Additionally, although the MTT assay is widely used, it measures only mitochondrial activity and does not capture all aspects of the cellular response, such as the release of inflammatory cytokines.<sup>[13]</sup> Therefore, future research should include complementary assays, *in vivo* studies, and long-term evaluations of newly developed multilayer aligners that combine PETG with polyurethane or other polymers.<sup>[9]</sup>

In conclusion, this study demonstrated that six commonly used thermoplastic aligner materials exhibited only slight cytotoxicity on HGFs, with outcomes influenced by material type, thickness, and thermoforming conditions. The observed time-dependent decline in viability underscores the importance of considering both material composition and clinical handling in aligner fabrication.<sup>[9]</sup> While current materials appear safe for clinical use, continued research is warranted to ensure optimal biocompatibility of next-generation aligners.

## CONCLUSION

The clear aligner materials that were tested showed only minimal cytotoxicity, with viability largely remaining within safe limits. Their use in clinical practice appears to be biologically safe, and careful material selection and ongoing innovation could further optimise patient outcomes.

**Ethical approval:** The research/study was approved by the Institutional Review Board at Sri Balaji Dental College, Hyderabad, approval number IEC/SBDC/PHD/ORTHO/0001-2022, dated 5th May 2022.

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