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In Vitro Cytotoxicity of Linseed Oil as Bio-Based Plasticiser in Polymethylmethacrylate (PMMA) Denture Base Material

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ABSTRACT

Objectives: Polymethyl methacrylate (PMMA) is a polymer that is vastly utilised as denture base material. Previously synthetic plasticiser like Dibutyl phthalate was previously used in the PMMA. However, there are reports of ester leaching causing intraoral toxicity. Thus, bringing the awareness about organic plasticiser usage, like linseed oil. This study aims to evaluate the cytotoxicity of linseed oil to ensure its safety to be used as the natural-based plasticiser in PMMA as denture base material.

Materials and Methods: The cytotoxicity of linseed oil was evaluated by culturing Human Gingival Fibroblast cells with 1, 3, 5, 7, and 10% linseed oil for 24, 48, and 72 hours. The WST-1 assay was then used to evaluate cell viability, with cell quantification performed using a spectrophotometer at a wavelength range of 420-480nm. An unpaired t-test was used to analyse all parameters' quantitative cell viability data.

Results: The WST-1 Assay demonstrated that linseed oil exhibited greater than 95% cell viability at all doses and incubation durations, indicating biocompatibility. At 72 hours of incubation, there are statistically significant differences between the cells incubated in the Linseed Oil as compared to Dimethyl Sulfoxide (DMSO), which acts as a positive control.

Conclusions: The study proves that linseed oil is non-cytotoxic with the Human Gingival Fibroblast cells and safe to use when incorporated later in the PMMA as denture base material.

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INTRODUCTION

Since the introduction of Polymethylmethacrylate (PMMA) in 1937, the material remains the most commonly used as a denture base (Alhareb et al., 2015) due to its excellent properties of low density, affordable, remarkable aesthetics and optimum mechanical and physical properties (Zafar, 2020). PMMA is a synthetic polymer formed by the free radical addition and polymerisation of methyl methacrylate ($C_5O_2H_8$) to polymethyl methacrylate ($C_5O_2H_8$) (Zafar, 2020).

Nonetheless, one of the significant areas for improvement is poor strength which accounts for many denture repairs each year (Saeed et al., 2020) because of the polymerisation shrinkage (Hassan et al., 2019). Therefore, various studies have been conducted on adding fillers to improve the mechanical and physical properties of the material (Gad et al., 2017; Saeed et al., 2020; Zidan et al., 2019). However, improving one set of properties is difficult without compromising the others.

Plasticisers like dibutyl phthalate were one of the components in PMMA that the manufacturers supplied to improve the water sorption properties of the PMMA (Kalachandra & Turner (1989). However, the leaching of ester of the dibutyl phthalate from the PMMA was found through in vitro study (Lygre et al., 1995) and in vivo study (Lygre et al., 1993) proven to be the cause of allergy-induced asthmatic, endocrine system disrupter, oestrogenic and carcinogenic properties and also hazardous to the environment (Haryono et al., 2017; Lithner et al., 2011). However, in vitro (Lygre et al., 1995) and in vivo (Lygre et al., 1993) research has shown that the leaching of the dibutyl phthalate ester from PMMA is the source of allergy-induced asthma, endocrine system disruption, oestrogenic and carcinogenic characteristics, as well as environmental hazards (Haryono et al., 2017; Lithner et al., 2011). Hence, the current PMMA in the market has excluded chemical plasticisers in their monomer. Thus, incorporating natural-based plasticisers like Linseed Oil into the PMMA as denture base material has come to our attention.

Linseed oil is one of the triglyceride vegetable oils (18.88% Oleic Acid, 16.10 % Linoleic Acid and 53.73 % Linolenic Acid) that is extracted from the flax plant (Linum usitatissimum). Linseed Oil, an edible oil (Goyal et al., 2014), was found to have excellent compatibility with polymethylmethacrylate by the ISO 9001: 2015, Industrial Specialties Mfg. & IS MED Specification and Ahmad et al. (2001) showed that adding the Linseed Oil into the PMMA resulted in a homogenous blend. Therefore, using Linseed Oil as a plasticiser was widely used in industries by adding polymers. Studies by Haryono et al. (2017), Navarchian et al. (2019), and Rahoma et al. (2020) showed that there were improvements in physical properties like heat and light stability and also plasticity of the polymer.

In medical fields, Çakmakli et al. (2007) suggested that a natural-based polymer with higher tensile strength was created by extracting PMMA copolymer from Linseed Oil (PLina-g-PMMA), and this supported the biocompatibility properties of Linseed Oil. Currently, there is no known study about incorporating natural-based Linseed Oil in PMMA as denture base material.

Hence, this study investigated Linseed Oil as a bio-based plasticiser compatible with polymethylmethacrylate (PMMA) by analysing the cytotoxicity effect of Linseed Oil on Human Gingival Fibroblast cells. The null hypothesis was that Linseed Oil is non-cytotoxic and can be incorporated well into the PMMA.

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MATERIALS AND METHODS

Cell Culture Media

The cells used for this study were Human Gingival Fibroblast (HGF) (ATC.CRL-2014, ATTC, US). These cells were grown as cultures in a 75 cm^2 vented cap tissue culture flask in the complete growth media of the Dulbecco's Modified Eagle Medium-Nutrient Mixture F-12 (DMEM/ F12) (Gibco, UK) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco, UK) and 1% (v/v) Penicillin-streptomycin (Pen/ Strep) (Gibco, UK).

Preparation of Linseed Oil for testing

Five different concentrations of Linseed Oil (Sigma Aldrich, US) were used in the study. For every concentration of Linseed Oil, 0.1% Dimethyl Sulfoxide (DMSO) (Sigma Aldrich, US) was used as the dilution with Linseed Oil before the addition into Basal Media, DMEM/ F12, as shown in Table 1. DMSO is a very efficient solvent for water-insoluble compounds and is frequently used in biological studies and as a vehicle for drug therapy (Santos et al., 2003).

Table 1. Percentages of Linseed Oil as Treatment

LOE Concentration (%)	Linseed Oil +DMSO (µl)	DMEM/ F12 (µl)
1	5	995
3	15	985
5	25	975
7	35	965
10	50	950

Five different percentages of DMSO were also prepared for incubation, as shown in Table 2. (Timm et al., 2013) showed that the usage of DMSO above 1% exhibited a cytotoxic effect on cell cultures. Hence, the 0.1% concentration was used in this study as the solvent for hydrophobic Linseed Oil, while 1,3,5,7,10 % concentration was used as the positive control in the study.

Table 2. Percentages of DMSO

DMSO Concentration (%)	DMSO (µl)	DMEM (µl)
1	10	990
3	30	970
5	50	950
7	70	930
10	100	900

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WST-1 cytotoxicity assay

The cytotoxicity of the specimens was analysed by WST-1 assay (Sigma Aldrich, US) (Francoeur & Assalian, 1996). This colourimetric assay is based on the cleavage of WST-1 tetrazolium salt via mitochondrial dehydrogenases (Konjhodzic-Prcic et al., 2015). The viability of cells was measured with the optical density of the formazan products.

Human gingival fibroblasts were seeded in a plate with 96 wells once the cell confluency achieved 80% of the 75 cm^2 flask. The number of cells was acquired by haemocytometer before 1000 µl complete growth media was added to each of the wells and were incubated for 24 hours in the incubator at 37 ^{O}C and 5% CO_2. The medium was removed when the human gingival fibroblasts became sub-confluent (Kim & Shin, 2014).

The seeded cells in the 96-well plates were then treated with five different concentrations of Linseed Oil 1%, 3%, 5%, 7%, and 10%, as mentioned in Table 1, with six duplicates for each concentration, including the control group. The control group (negative control) was treated with Basal Media (DMEM/ F12) with no addition of Linseed Oil. The cells were then stored in the incubator for 24, 48 and 72 hours at 37 ^{O}C and 5% of CO_2. After the incubation period, the WST-1 cytotoxicity assay was performed.

At the end of the process, 10 µl WST-1 was added and incubated for 2 hours at 37 C and 5% CO_2. The absorption of cells was read with a spectrophotometer plate reader (Tecan Infinite M200 Plate Reader, Switzerland) at 420-480 nm.

For the viability of cells, the formula as shown below was used:

$$\% \text{ Viability of cells} = [(\text{mean absorbance of treated cells}) - (\text{mean absorbance of blank cells}) / (\text{mean absorbance of control cells}) - (\text{mean absorbance of blank cells})] \times 100 \quad (1)$$

Statistical analysis

The mean absorbencies and the standard deviation of the percentages of cell viabilities in the six wells duplicates for each concentration of Linseed Oil, and DMSO were calculated. Cytotoxicity was rated based on cell viability relative to controls as non-cytotoxic ; > 90% cell viability, slightly cytotoxic- 60-90 % cell viability, moderately cytotoxic; 30-59% cell viability and strongly cytotoxic; <30% cell viability (Konjhodzic-Prcic et al., 2015). An unpaired t-test was used to analyse the quantitative significance level for all parameters using the Prism Software (GraphPad Software, Inc) version 9.0.0 for MacOS.

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RESULTS AND STATISTICAL ANALYSIS

Table 3. Mean and Standard Deviation of Cell Viability for Different Concentrations of Linseed Oil and Dimethyl sulfoxide (DMSO) according to the treatment hours.

Concentrations (%)	Treatment (hours)	Linseed Oil		DMSO	
		Mean	Standard Deviation	Mean	Standard Deviation
Control (0%)	24	100	0	100	0
	48	100	0	100	0
	72	100	0	100	0
1	24	99.722	1.537	102.541	4.883
	48	94.798	0.991	87.329	10.164
	72	98.541	3.890	86.303	6.553
3	24	100.519	3.141	103.466	3.932
	48	96.8157	2.837	89.559	9.835
	74	100.241	3.180	83.413	5.592
5	24	100.047	2.452	99.408	2.939
	48	96.574	3.473	88.972	9.159
	72	101.148	0.412	74.524	7.105
7	24	101.739	2.955	94.664	3.294
	48	95.192	4.529	84.747	10.046
	72	98.389	0.727	70.834	6.152
10	24	102.458	6.374	93.273	4.741
	48	96.555	3.410	84.246	10.112
	72	99.669	1.006	68.459	6.068

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Table 4. The unpaired t-test between the six concentrations of Linseed Oil and DMSO within 24, 48 and 72 treatment hours.

Treatment time (hours)	N	Standard Deviation	95% Confidence Intervals		p-value
			Lower Bound	Upper Bound	
24	6	1.537	-5.741	2.030	0.3124
48	6	0.991	-12.99	-2.033	0.0121
72	6	3890	-29.85	-8.297	0.0028

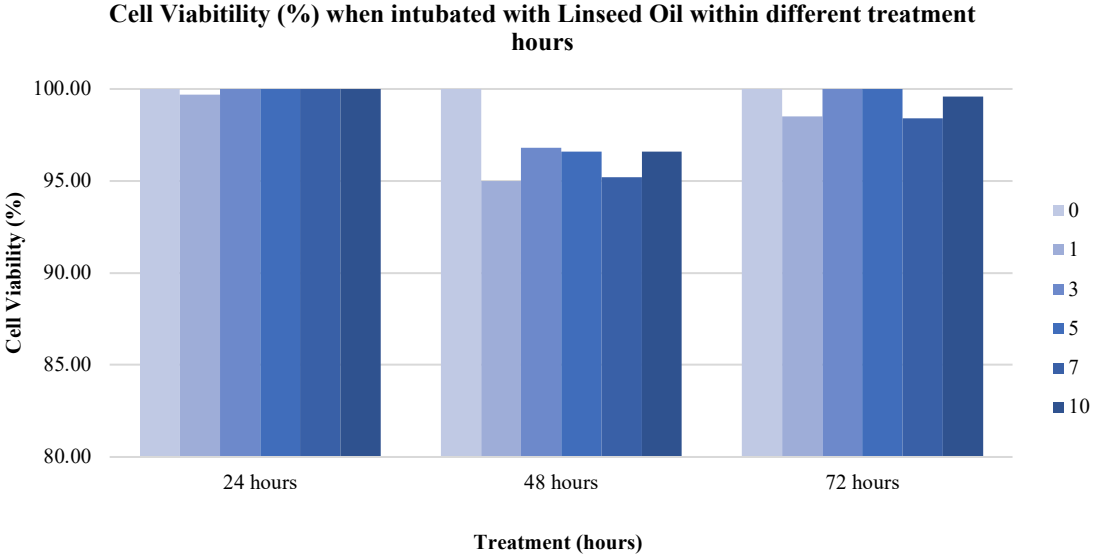

Fig. 1. The Percentage of HGF Cell Viability Count in five concentrations of Linseed Oil and DMSO within 24, 48 and 72 hours.

Overall, the study found that Linseed Oil showed no negative impact on cell viability, while Dimethyl Sulfoxide (DMSO) showed a decrease over time. The most significant disparity was observed at the 10% Linseed Oil concentration. A statistically significant distinction was also found between Linseed Oil and DMSO at 72 hours but not at 24 or 48 hours.

Table 3 presents the mean percentage of cell viability in five different concentrations of Linseed Oil (1%, 3%, 5%, 7%, and 10%) and DMSO at three different incubation periods (24, 48, and 72 hours). In the control group, 100% cell viability was observed for both Linseed Oil and DMSO. The percentage of cell viability in the Linseed Oil group remained consistently above 94% at all incubation periods until 72 hours, indicating no adverse effects on HGF cells exposed to any concentration of Linseed Oil (Figure 1).

However, in the DMSO group, the mean percentage of cell viability decreased even at the lowest concentration of 1% after 24 hours of incubation (102.541%) to 86.303% at 72 hours. Similarly, a significant reduction was observed with 10% DMSO between 24 hours of incubation (93.273%) and 68.459% at 72 hours.

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The data for the percentage of cell viability, treated with different concentrations of Linseed Oil and Dimethyl Sulfoxide (DMSO), were assessed, and found to be normally distributed. An unpaired t-test was then performed. Table 4 demonstrates a statistically significant difference in the percentage of cell viability at 72 hours of incubation between the groups incubated in all concentrations of Linseed Oil compared to DMSO (p-value = 0.0028, $p<0.05$). However, there was no statistically significant difference in the incubation period at 24 hours (p-value > 0.05, p-value = 0.3124) and 48 hours (p-value > 0.05, p-value = 0.01).

DISCUSSION

The study on cell viability is crucial to provide information on the efficacy and safety of a material before its usage intraorally. Multiple assays are available to assess cell viability, including the most common ones are the Trypan blue exclusion assay, MTT assay and the WST-1 assay (Stoddart, 2011). Trypan blue is a dye that can distinguish between live and dead cells. Live cells with intact cell membranes exclude the dye, while dead or dying cells take up the dye, appearing blue under a microscope. In comparison, the MTT assay measures the activity of mitochondrial enzymes in viable cells. These enzymes convert the MTT reagent into a coloured formazan product, which can be quantified spectrophotometrically.

On the other hand, this study evaluated the effects of different concentrations of Linseed Oil on the percentage of cell viability of Human Gingival Fibroblast cells via WST-1 assay. Given its sensitivity and accuracy, WST-1 assay was used to evaluate cell cytotoxicity and cell proliferation and estimate the number of viable cells in cultures. The assay measured the transformation of the tetrazolium salt WST-1 to formazan by cellular dehydrogenases. The formazan generated was dark yellow, measured at 450nm and was directly correlated to cell number (Sarı, 2019).

From the above results, we observed an insignificant influence of the percentages of Linseed Oil towards the percentage of cell viability of the Human Gingival Fibroblast (HGF) cells. Therefore, the null hypothesis was accepted: Linseed Oil is not cytotoxic to HGF cells. Figure 1 shows that the incubation time from 24 to 72 hours had no significant impact on cell viability. Cell viability stayed above 94% of all the Linseed Oil concentrations with no significant changes even at 72 hours. The result indicated that Linseed oil is not cytotoxic to Human Gingival Fibroblast, as supported by Sjögren et al. (2000). Their study found that the medium is categorised as non-cytotoxic if the cell viability is greater than 90%. Table 2 shows a statistically significant difference between the cells incubated in Linseed Oil at 72 hours compared to Dimethyl Sulfoxide ($p<0.05$, p-value = 0.0028). Lee & Park, 2017 supported this result with the findings in their study, which showed that the treatment with higher concentrations of DMSO at 3 and 5% resulted in a noticeable decrease in cellular viability.

The result of cell viability at all concentrations of Linseed Oil was also in compliance with the status Generally Recognised As Safe (GRAS) appointed by the United States Food And Drug Administration (FDA) for Linseed Oil, which means that it is safe for applications and consumptions at levels of 1.0 – 10.0%. Lewinska et al. (2015) reported that Linseed Oil is the type of edible oil utilised as a functional dietary component with multiple health advantages, including management of cardiovascular diseases, diabetic therapy, and anti-inflammatory and autoimmune disorders (Goyal et al., 2014; Rabail et al., 2021).

Considering only one test method to determine cell viability values of Linseed Oil in this study can be seen as a limitation. Employing multiple test methods could provide more dependable outcomes. Furthermore, in vitro test methods have various limitations regarding their direct correlation with clinical scenarios. Although the study's findings have provided insights into the toxicity of the substances utilised, conducting in vivo studies is essential to garner insights into definite consequences.

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CONCLUSION

This study investigated the effects of Linseed Oil's different concentrations on the cell viability of Human Gingival Fibroblast cells. Within the limitations of this study, it was found that the Linseed Oil is biocompatible and non-cytotoxic against human gingival fibroblast cells and safe to be incorporated in the polymethylmethacrylate (PMMA) as denture base material.

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CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare the absence of conflicting interests with the funders.

AUTHORS' CONTRIBUTIONS

Nadiyah Abdullah Zawawi carried out the research, wrote and revised the article. Hazlina Abdul Ghani and Farrah Hazwani conceptualised the central research idea and provided the theoretical framework. Raja Azman Raja Awang and Nik Noor Idayu Nik Ibrahim designed the research, supervised research progress; Hazlina Abdul Ghani anchored the review, revisions and approved the article submission.

ABBREVIATIONS

PMMA: Polymethylmethacrylate

DMEM/ F12: Dulbecco's Modified Eagle Medium-Nutrient Mixture F-12

DMSO: Dimethyl Sulfoxide

HGF: Human Gingival Fibroblast

ISO: International Organization for Standardization

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