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Evaluation of Radio-opacity, Setting Time and Cytotoxicity of Different Root Canal Sealers

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Abstract

The aim of this study was to evaluate radio-opacity, setting time and cytotoxicity of commercially available root canal sealers (Tech Biosealer Endo and MTA-Fillapex)and Flavonoid-based experimental sealer. The radiopacity of each material was determined using an aluminium step-wedge and densitometer, while Initial and final setting time was evaluated using Gilmore needle system. Cytotoxicity was evaluated by MTT assay to check the human fibroblasts cells viability at 24 and 72 hours periods. Mixed ANOVA and Univirate ANOVA were used to assess effect of time and sealer over cytotoxicity, and setting time. A one-way Analysis of Variance (ANOVA) was used to comparison between sealers regarding radiopacity followed by Tukey's post-hoc test. The significance level was set at $P \leq 0.05$. Tech Biosealer Endo showed mean radio-opacity value comparable to MTA-Fillapex, while Flavonoid-based experimental sealer has mean radio-opacity value significantly lower than other tested sealers ($p<.05$). Flavonoid-based experimental sealer has shown significantly longest mean initial and final setting times followed by MTA-Fillapex and Tech Biosealer Endo ($p<.05$). Finally, for all observation periods, the significantly highest cytotoxicity was exhibited by MTA-Fillapex ($p<.05$). The present study concluded that, both Tech Biosealer Endo and MTA-Fillapex sealers have physical properties(radio-opacity, setting time) in agreement with ISO and ANSI/ADA specification , while Flavonoid-based experimental sealer and Tech Biosealer Endo were still superior to MTA-Fillapex regarding cytotoxicity.

Keywords: *MTA-based root canal sealers, physical properties, cytotoxicity, MTT assay.*

1. Introduction

For successful endodontic treatment, fostering a leak-proof apical seal throughout the canal system after biomechanical preparation is crucial. In order to achieve this hermetic seal, a combination of endodontic sealer and gutta-percha are used. Gutta-percha is widely used because of its good physical and biological properties, but the lack of adhesiveness and flow makes the association with endodontic sealers necessary. According to Grossman (1), an ideal root canal

sealer should provide the following: an excellent seal when set, dimensional stability, a slow setting time to ensure sufficient working time, insolubility to tissue fluids, adequate adhesion with canal walls, and biocompatibility. From function of root canal sealer, the radiopacity of sealer may help to disclose presence auxiliary canal, resorption area or root fracture. Also the sealer should be provide a fluid-tight seal at the apex, fill of irregularities, accessory canals and minor spaces between the solid core & the canal walls. Propolis is a resinous material collected by honey bees from various plant species. It has attracted increased interest due to its bioactivities such as anti-inflammatory, anti-tumor and antimicrobial activity against a wide range of pathogenic microorganisms. The precise composition of raw Propolis varies with the source. however the Flavonoid has been considered as the main primary biologically active component. Calcium silicate cements, well known as MTA. They have received greater attention in endodontics because they are able to set in presence of biological fluids, and their biocompatibility. They appeared interesting to develop endodontic sealers based on calcium silicate hydraulic cements. Tech Biosealer Endo is a recently introduced MTA-based sealer. It is become important to evaluate the characteristics of this sealer as any new dental product must be tested before being cleared for clinical use. MTA-Fillapex is another MTA-based sealer. This sealer has been extensively evaluated for its physicochemical properties and biological response. The aim of this study was to estimate some physical properties and cytotoxicity of Propolis to be used as endodontic sealer (Flavonoid-based experimental sealer) and compare it to the newly introduced Tech Biosealer Endo in addition to MTA-Fillapex sealer.

2. Materials and Methods

Specimen Preparation

Tech Biosealer Endo (Isasan SRL, Rovello Porro, CO, Italy), MTA-Fillapex (Angelus, Londrina PR, Brazil) and Flavonoid-based experimental sealer (Imtenan Health Shop, Cairo, Egypt), were used as an experimental materials. All sealer were mixed and manipulated in accordance with the manufacturer's instructions except for the *Flavonoid-based experimental sealer*; in which 6 g of Propolis powder was placed in container, then 20 ml of 70% solution of ethanol was poured into container and shake briefly. The mixture in well-sealed container was stored at a warm and dark place for one week. Repeat the shaking of container once or twice per day during soaking period. After two weeks, ethyl alcohol was partially evaporated by gently heating of the mixture with an open container in water bath for approximately 30 minutes at low heat (not more than 60 ºC). The evaporation was continuing until obtained homogeneous product with paste-like consistency (2). Immediately after obtained the creamy mix consistency, the paste placed in different molds to subject the different tests. Forty eight discs were divided into three

equal main groups according to the tested material; Group I*:* 16 discs from Tech Biosealer Endo. Group II: 16 discs from Flavonoid-based experimental sealer. Group III: 16 discs from MTA-Fillapex. Each group was further subdivided into 2 subgroups according to the evaluation test as follows; Subgroup A: used to evaluate the radiopacity of the sealer (8 discs). Subgroup B: used to evaluate the setting time of the sealer (8 discs). For cytotoxicity, forty five discs (15 each sealer) were subdivided into 2 subgroups for evaluation of cytotoxicity at 24 and 72 hours.

Radio-opacity Evaluation

Stainless steel ring molds were fabricated with dimensions 10mm internal diameter and 1mm thick (3,4). One sample from each sealer was placed on the digital sensor (Kodak, Rochester, NY, USA) adjacent to a 99% aluminum step-wedge (Agfa Mamoray, Agafa Gevaert, Mortsel, Belgium), with thickness graduated from 1 to 10 mm (each step measured 1mm in height). Digital radiograph images were obtained by using periapical x-ray machine (Kodak, Rochester, NY, USA.) operating at 65 KV and 10 mA, for 0.3 seconds of exposure time, and object-to-focus distance of 30 cm (5, 6, 7, 8). The exposed sensor was immediately scanned by Digora Scanner, and Digora software (Soredex, Helsinki, Finland) to identify density as the degree of whiteness of an image. The image consists of pixels; each has a certain density value between zero and 255. The density value zero represents black and 255 represents white. The different shades of gray vary from one to 255. To convert the mean pixel intensity into radio-opacity values, fixed region of interest in each step of the step-wedge were measured to determine the mean gray level of each step on every radiograph. By recording the gray levels of pixel intensity of both step-wedge and the disc, the thickness of aluminum equivalent to radio-opacity of the disc could be estimated.

Setting Time Evaluation

Stainless steel ring molds were fabricated with dimensions 10mm internal diameter and 2mm thick (3, 4). Immediately after mixing of each sealer, the molds were filled with the sealers and then fixed on a glass plate. The setting time of each sample was tested using a Gilmore needle (Gilson Company, Inc. Ohio) according to the C266-03 specification of the American Society for Testing and Materials (ASTM) (8,9). At 150± 10 seconds after the onset of mixture, each specimen was indented with Gilmore-type needle with a mass of 100 ± 0.5 g having a flat end 2.0 ± 0.1 mm in diameter for measuring the initial setting time. After the initial setting time was measured, another Gilmore-type needle with a mass of 456 ± 0.5 g having a flat end 1.0 ± 0.1 mm in diameter was used to measure the final setting time. The Gilmore needle was carefully lowered vertically onto flat surface of the sealer. The indenter is wiped clean between indentations. The setting time was calculated by measuring the time elapsed between the start of mixing and the time when no indentation is visible on the sealer surface.

Cytotoxicity Evaluation

Cell Cultures

An established cell line, human fibroblasts, MRC-5 (American Type Culture Collection ATCC. Manassas, VA, USA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mm L-glutamine and Earle's balanced salt solution (EBSS) adjusted to contain 90% 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 Mm sodium pyruvate; and 10% fetal bovine serum (FBS). The cultures were maintained at 37°C in humidified atmosphere (5% Co2, 95% air) (10).

Extraction Procedures

Discs of each sealer were placed in culture tubes, and cell culture medium (Dulbecco's Modified Eagle's Medium, DMEM) was added using the ratio 1.25 cm^2/ml between the surface area of the sealer samples and the volume of medium (11). The samples were incubated in culture medium for 24 hours at 37°C to allow the soluble materials to leach from the samples into the medium. The medium was filtered through 0.2 µm syringe filters (Millipore, Bedford, MA, USA) to remove particular matter before use. The extracts obtained were then incubated at 37°C for 7 days. Control samples containing only the medium were incubated similarly. Each extraction media were then collected using a Millex-GS sterile filter (Millipore SAS, Molsheim, Cedex, France). Several dilutions of the extraction media were obtained using Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) to achieve a total of eight concentrations (Neat, 1/2, 1/4, 1/8, 1/16,1/32, 1/64 and 1/128 V/V).

MTT Assay

The MTT assay is a simple colorimetric assay developed by Mosmann (12) and modified by Edmondson et al (13) was used as a test for cell proliferation and survival in this part of the study. Cytotoxicity was measured using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye solution (MTT)]. Human fibroblasts, MRC-5(1.0×104 cells/well) were seeded into 96-well plates, incubated in a humidified atmosphere of 5% CO2 at 37°C for 24 hours, and then exposed to the extract of the test material for 24 hours and 72 hours. After incubation, the medium was removed, the cells were washed with phosphate buffered saline (PBS), and 0.2 ml of the medium and 20 microl of MTT solution (5 mg of MTT/ml PBS) were added to each well. The MTT was aspirated and the formazan product was solubilized in 50 microl dimethyl sulphoxide (DMSO). The plates were shaken before the optical densities were measured at 570 nm, using an enzyme-linked immunesorbent assay (ELISA) plate reader (Dynatech-MRX, CA, USA). All assays were repeated at least twice to ensure reproducibility. The absorption value obtained with the control was deemed to indicate 100% viability. The percentage of viable cell was calculated using the

following equation:

Percentage of Viable Cells = (A / B) × 100

Where A = viable cells in the experimental well and

B = viable cells in the control well

3. Results and Discussion

Radio-opacity

The mean radio-opacity value was 6.95±0.339 mm Al in the Tech Biosealer Endo group, 1.87±0.572mm Al in the Flavonoid-based experimental sealer group and in MTA-Fillapex group was 6.53±0.291 mm Al. There was no statistically significant difference between Tech Biosealer Endo and MTA-Fillapex, while there was a significant difference between Flavonoid-based experimental sealer and other tested sealer ($p<.05$).

Initial and Final Setting Time

The mean initial setting time was 40±3.741 minutes in Tech Biosealer Endo group, 1320±27.902minutes in Flavonoid-based experimental sealer group and in MTA-Fillapex group 135 ±8.734 minutes. The mean final setting time was 70±4.898 minutes in Tech Biosealer Endo group, 10080±0.000 minutes in Flavonoid-based experimental sealer group and in MTA-Fillapex group 295±10.212 minutes. The mean initial and final setting time of the Flavonoid-based experimental sealer was showed longest initial setting time followed by MTA-Fillapex then Tech Biosealer Endo with a significant difference between all groups ($p<.05$). All sealers showed a significant difference between the initial and final setting times ($p<.05$).

MTT Assay

Effect of Sealer on Cell Viability at Different Examination Period

The mean, standard deviation values and results for comparison between cell viability percentage with different dilutions after 24 hours and 72 hours were presented in (Table 1 and 2). MTA-Fillapex was significantly higher cytotoxic than Tech Biosealer Endo and Flavonoid-based experimental sealer ($p<.05$), especially at the high extract concentrations (Neat,1/2, 1/4, 1/8 dilutions).

Effect of Time on Cell Viability of Each Sealer

The mean, standard deviation values and results for comparison between cell viability percentage with different dilutions in Tech Biosealer Endo group, Flavonoid-based experimental sealer group, and MTA-Fillapex group were presented in (Fig. 1, 2, 3).

Table 1. The mean, standard deviation (SD) values and results of one –way ANOVA for comparison between cell viability percent with different dilutions after 24 hours.

Subgroup / Dilution	Tech Biosealer Endo (IA)		Flavonoid- sealer (IIA)		MTA-Fillapex (IIIA)		*P-value*
	Mean	SD	Mean	SD	Mean	SD	
1/1	53.7 [a]	3.3	41.5 [b]	3.5	22.6 [c]	0.9	<0.001*
1/2	66.3 [a]	2.1	71.0 [a]	6.1	30.7 [b]	1.6	<0.001*
1/4	101.1[a]	4.4	93.9 [a]	17.1	36.7 [b]	2.1	<0.001*
1/8	101.5[a]	21.1	103.5[a]	21.6	45.9 [b]	1.4	0.002*
1/16	100.8 [a]	24.1	97.6 [a]	14.2	101.0 [a]	7.1	0.949
1/32	100.5 [a]	20	95.08 [a]	13.6	102.1 [a]	11.5	0.8
1/64	100.4 [a]	6.5	104.8 [a]	18	99.9 [a]	14.1	0.858
1/128	98.3 [a]	18.7	97.6 [a]	17.6	101.4 [a]	9.2	0.937

Same small letters within the same row indicate non-significant difference

*; significant at $P \leq 0.05$.

Table 2. The mean, standard deviation (SD) values and results of one –way ANOVA for comparison between cell viability percent with different dilutions after 72 hours.

Subgroup / Dilution	Tech Biosealer Endo (IB)		Flavonoid-based (IIB)		MTA-Fillapex (IIIB)		*P-value*
	Mean	SD	Mean	SD	Mean	SD	
1/1	24.8 [a]	0.33	26.7 [a]	2.91	20.6 [b]	1.46	<0.001*
1/2	33.8 [a]	4.015	34.0 [a]	1.00	32.1 [a]	1.26	0.298
1/4	61.3 [a]	7.10	63.4 [a]	4.00	35.9 [b]	1.83	<0.001*
1/8	66.3 [a]	4.90	72.5 [a]	15.40	42.3 [b]	3.23	0.004*
1/16	95.5 [a]	14.19	86.1 [a]	43.27	71.1 [a]	4.81	0.115
1/32	98.8 [a]	4.63	97.5 [a]	11.62	95.0 [a]	5.65	0.791
1/64	97.7 [a]	14.67	96.2 [a]	7.52	96.0 [a]	4.95	0.967
1/128	101.6 [a]	7.10	102.2 [a]	10.72	102.4 [a]	5.19	0.99

Same small letters within the same row indicate non-significant difference

*; significant at $P \leq 0.05$

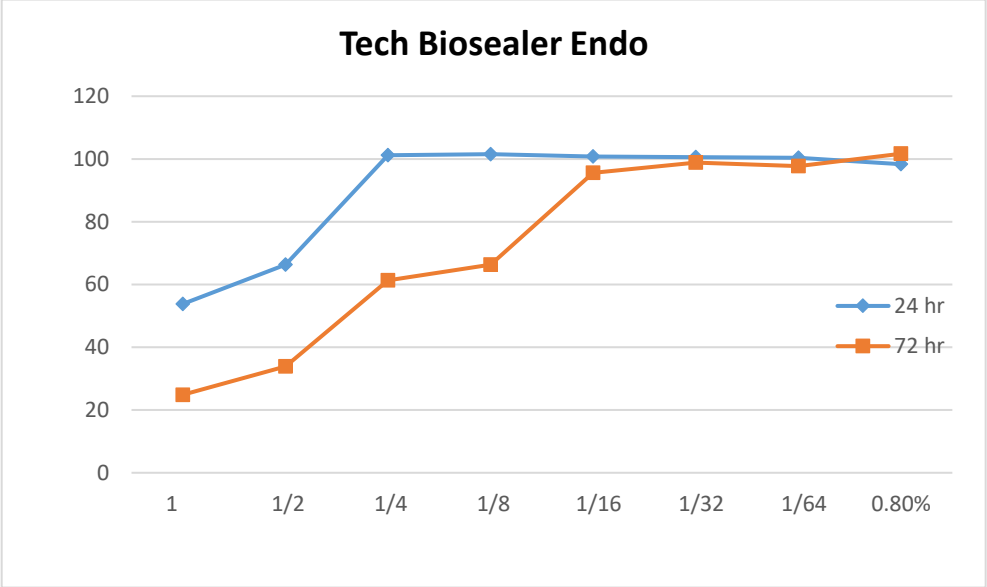

Figure 1. The mean cell viability percent with different concentrations in Tech Biosealer Endo group.

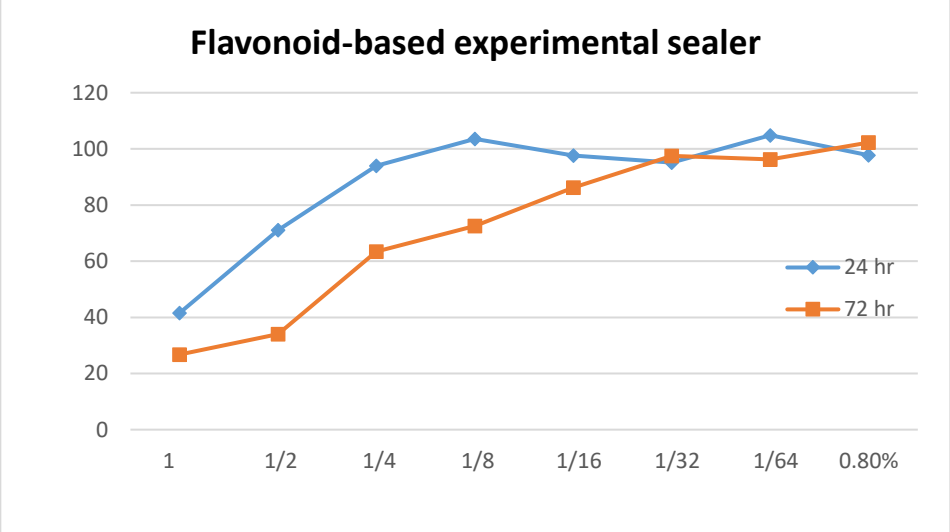

Figure 2. The mean cell viability percent with different concentrations in Flavonoid-based sealer group

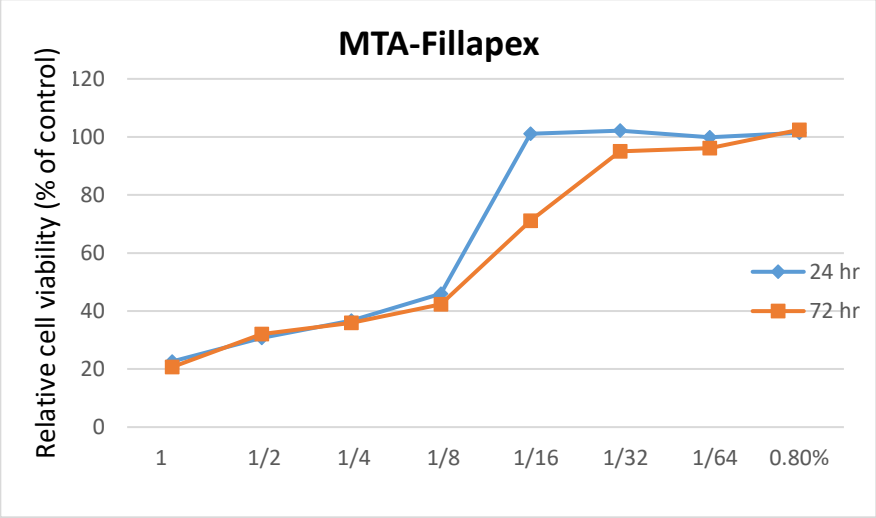

Figure 3. The mean cell viability percent with different concentrations in MTA-Fillapex group.

New sealers are constantly being developed in attempts to provide favorable properties. The properties of an ideal root canal sealer include adequate working time, dimensional stability, adequate radiopacity, low solubility, creating a bacteria-resistant seal, possessing antimicrobial activities, being tissue tolerant, and providing good adhesion with the radicular dentin (14). Radiopacity is an essential property of endodontic sealing materials. The ideal root canal sealer should have a certain degree of radiopacity to be clearly visible on radiographs and enhance the radiopacity of the root filling materials. MTA without radiopacifying additives have intrinsic radiopacity values ranging from 0.86 to 2.02 mm Al (15), values lower than the 3 mm Al recommended by the international standards for dental root canal sealing materials. Thus, a radiopacifying material has to be added to calcium silicate-based cements. In addition, the setting time of a sealer is important to allow adequate working time and proper consistency to permit complete filing of the root canal system. Cytotoxicity was evaluated using MTT assay. MTT is a colorimetric method based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble methyl-thiazol-tetrazolium salt (MTT) into dark-blue formazan crystals. The amount of formazan produced is directly proportional to the total viable cell number because dead cells are unable to produce the colored formazon product (12).The advantages of this method are its simplicity, rapidity, and reliability. In addition, it does not require radioisotopes.

In the current study, the radiopacity values for Tech Biosealer Endo and MTA-Fillapex were statistically significantly higher than that of Flavonoid-based experimental sealer. Results of both Tech Biosealer Endo and MTA-Fillapex were found to be in agreement with ISO 6876/2001

recommendations (4). However the radiopacity value of the Flavonoid-based experimental sealer was less than the standard value compared with that of 3mm thickness of aluminum. So, it does not meet the minimum requirement for radiopacity set out in ISO 6876;2001(4). A radiopacifier should be added to Propolis to enable it to be used clinically. The differences between radiopacities of the Tech Biosealer Endo and MTA-Fillapex in the present study may be caused by the presence of different percentage of the same radiopacifying agent (bismuth oxide) in both sealers. Results of the present study are in agreement with previous studies (7, 16), which shown the radiopacity of MTA-Fillapex to be in agreement with ISO 6876/2001 recommendations (4). Tech Biosealer Endo has a 20% loading of bismuth oxide (17). The amount of bismuth oxide present in MTA-Fillapex is not specified by the manufacturer, however presence of 20% bismuth oxide in calcium silicate cements produced the same radiopacity required by ISO 6876/2001 recommendations (5). In this study, Tech Biosealer Endo showed shortest initial and final setting time, while Flavonoid-based experimental sealer showed longest initial and final setting time with a statistically significant difference between all groups. Tech Biosealer Endo is powder to liquid material, when calcium silicate particle of MTA react with water, tricalcium silicate and dicalcium silicate produce a calcium silicate hydrate gel (CSH) and calcium hydroxide during hydration reaction. The colloidal gel solidifies to a hard structure. in the present study, the short initial and final setting time of Tech Biosealer Endo may be caused by the presence of calcium chloride (CaCl2) as setting accelerator in its composition. This results are in full agreement with an earlier study (18). In that study, 10% and 15% CaCl2 were added to MTA and Portland cement, found that the presence of CaCl2 was responsible for shorter setting time with respect to the other tested cements. Another study by Kogan et al (19) focused on the effects of additives on setting properties of MTA reported that CaCl2reduced the setting time. Results of the present study are in full agreement with Vitti et al (9) who studied the initial and final setting times of MTA-Fillapex. Initial setting time results of MTA-Fillapex came also in agreement with those of Zhou et al (20). Initial and final setting times of Tech Biosealer Endo came in agreement with those of Gandolfi et al (21). The final setting time of Tech Biosealer Endo obtained in this study was in full agreement with Prati and Gandolfi (17). Our results could not be compared to those of Prati et al (22), due to the different methodology used. In that study, they evaluated the initial and final setting time of Tech Biosealer Endo under immersion in simulated body fluid.

In the present study, MTA-Fillapex displayed the highest cytotoxic rates in a dose-dependent manner and the viability decrease caused by MTA-Fillapex exceeded 75% when pure and ½ diluted. This severe cytotoxicity of MTA-Fillapex did not decrease over the tested time periods. MTA-Fillapex was significantly higher cytotoxic than Tech Biosealer Endo and Flavonoid-based experimental sealer, especially at the high extract concentrations (Neat,1/2, 1/4, 1/8 dilutions). The addition of salicylate resin, diluting resin, bismuth oxide and other pigments to components of MTA-Fillapex may have increased the toxic effect of this sealer compared to its precursor, MTA.

Also, the material with low MTA content become incapable to release favorable amounts of Ca+ ions, to provide the biocompatibility. These results came in full agreement with previous in vitro studies (7, 23, 24, 25, 26). These studies showed that the cell viability was strongly affected with MTA-Fillapex. Tech Biosealer Endo and Flavonoid-based experimental sealer showed less cytotoxicity to fibroblast cells compared with MTA-Fillapex . At 24 hours, the diluted extracts of both sealers were found to be slight or not cytotoxic under the condition of the present study. In the current study, results of Tech Biosealer Endo came in agreement with Hakki et al (27) who found an increase in cell viability after exposure to concentration 1/4 of Tech Biosealer Endo at 24hours, while concentration 1/2 decreased cell viability. They also found that the fibroblast viability of Tech Biosealer Endo decreased with increasing observation time. Results of this study could not be directly compared to those results obtained by Khedmat et al (28) who found that Tech Biosealer Endo have significantly high cytotoxic effect on monocyte cells compared to ProRoot MTA and Biodentine, due to different in cell type and compared materials. Cytotoxic results of Flavonoid-based experimental sealer came in agreement with Al-Shaher et al (29) who treated the fibroblasts of the pulp and periodontal ligament with Propolis. They concluded that this material is not toxic. Silva et al (30) compared biocompatibility of Propolis with other experimental materials and found that Propolis was the least irritant one which can make it a valuable alternative material for endodontics treatment. These results were in accordance with our findings. MTT results in the present study had demonstrated that the pure-1/16 concentrations of Flavonoid-based experimental sealer were cytotoxic for human fibroblasts. This was in accordance with Sonmez et al (31).

Conclusion

Under the condition of the present study, it can be concluded that:

1. Both Tech Biosealer Endo and MTA-Fillapex sealers have physical properties in agreement with ISO 6876/2001 and ANSI/ADA specification no. 57. while the radio-opacity of Flavonoid-based experimental sealer does not conform to the requirements of both ISO and ANSI/ADA specifications. Flavonoid-based sealer also showed long initial and final setting time.
2. Tech Biosealer Endo and MTA Fillapex, the 2 MTA based root canal sealers, exhibited different cytotoxicity to human fibroblasts. Flavonoid-based experimental sealer and Tech Biosealer Endo are still superior to MTA-Fillapex regarding cytotoxicity. Severe cytotoxic results were detected with MTA Fillapex over three days.

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