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Cytotoxicity and Electrochemical Study the Effect of ALF3 Incorporation to Acrylic Resin Materials

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KEYWORDS
Acrylic resin ,mtt assay AlF3, Artificial Saliva, pH

ABSTRACT:

Aluminum fluoride (AlF3) was one of the compound that treated with different denture composition, also used incorporation with acrylic resin to improve and support the physical and chemical properties.

Aim: to find the electrochemical analysis by cyclic voltammetric (CV) technique, the physical properties of these material and the effected in artificial saliva (AS) by oxidation – reduction peak current in oral cavity. Also to Evaluate the cytotoxicity effect of ALF3 incorporation to acrylic resin materials

Material and method: for electrochemical analysis used glassy carbon electrode (GCE) as working electrode, Ag/AgCL as reference electrode, and platinum wire as axillary electrode in cyclic voltammetric cell. Different chemical properties such as concentration, pH, scan rate, and reproducibility study were used in potentio-state.

Ten samples incorporated with different concentration of different fluoride salts as follow: 2%AlF3 and 5%AlF3 ,five samples for each concentration, all these specimen cultured for 24h,48h and 72h.and test for mtt test).

Results: Oxidation peak current was appeared in the voltammogram of AlF3 in artificial saliva at 125 mV which was studied the electrochemical behavior at different ways. The oxidation peak was gradually enhanced by increasing the concentrations and scan rates that give a calibration curve with high sensitivity, but in different pH the oxidation peak of AlF3 was disappeared in alkaline pH and enhanced in acidic medium, so the AlF3 ions act as oxidative reagent in artificial saliva, and antioxidant in alkaline pH, so the low concentration of 2%AlF3 in the acrylic was more favor than high concentration also addition of 2% and 5% AlF3 to heat cured acrylic resin materials showed no effect on the cytotoxicity test.

Conclusion: In the high concentration of 5%AlF3 has been affected in oxidation behavior more than low concentration of 2%AlF3. In the acidic pH of the artificial saliva has high intensity of oxidative property which act damage to the cell of mouth while in the alkaline pH of artificial saliva act as antioxidant behavior that can be used in safety .The addition of 2% and 5% of AlF3 into heat cured acrylic resin materials showed no toxic effect.

1. Introduction

A method was developed to identify the extent of knowing the toxicity effect of the chemicals and pharmaceuticals compounds using electrochemical analysis by cyclic voltammetry technique to finding the oxidative or antioxidant [1,2,3,45].

Some studies were determined the amount of ALF3 released from different restorative materials in artificial saliva and distilled water [6,7,8].

Various studies have shown that prolonged exposure to fluoride (F^-) and aluminum (Al^{3+}) ions is associated with several diseases including neurological disorders. They have no known biological function. But they can bind to proteins that interact with ions that are similar to them. These undesirable interactions affect the normal biological function of target proteins, as well as protein-protein interactions downstream.[9]

Bending strength, flexural modulus, and the amount of

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fluoride released from four experimental denture base resins containing 5, 10, 20, and 30% by weight of a pre-reactive glass filler (S-PRG) added to the powder were evaluated. The average flexural strength of the experimental resins, except for 30 wt%, and the flexural modulus of all resins, comply with ISO 1567 requirements. In the 20 wt% resin, the amount of fluoride released in the initial phase was 1.88 μg/cm2/day, after which the level decreased. After recharging with a 9000 ppm fluoride solution for eight hours, the level of released fluoride increased significantly to 40.21 μg/cm2/16 hours, the results show that fluoride levels increased as a function of S-PRG filler content. These results indicate that denture base resins containing S-PRG fillings have remarkable recharge and release capabilities that may help prevent root caries of the abutment teeth.[10]

The role of exogenous fluoride (F-) in relieving aluminum (Al) stress in plants has not yet been investigated. Study , barley (Hordeum vulgaris) seedlings were exposed to increased concentrations of Al3+ (aluminum chloride, 0.5, 1.0, 2.0, 3.0, 4.0 mM) with and without fluoride (0.025% NaF) to explore potential roles of fluoride in mitigating toxicity. [11]

A new method has been developed for the determination of fluoride at levels with good precision and accuracy using differential pulse polarimetry. It is based on wave dip Polarography to reduce uranium (VI) by fluoride ions. A peak linear elevation of fluoride concentration was found between 0.08 to 20.00 μg/mL. The limit of determination of 0.08 μg/ml was achieved. The proposed method has been successfully used for the determination of fluoride in waste water, tap water, table salt and tea leaves.[12].

The Mosmann's Tetrazolium Toxicity assay (MTT) is a quantitative
cytotoxicity assay that uses a dye called (3-[4,5-dimethylthiazol-2-yl]-2,5-
diphenyl tetrazolium bromide) is a water soluble tetrazolium salt, which is
converted to an insoluble purple formazan (13,14) by cleavage of the tetrazolium ring by succinate dehydrogenase enzymes within the mitochondria. This reaction will only occur in health living cells because of dehydrogenase enzymes only present in viable cells
which reduce the yellow MTT to purple insoluble formazan crystals which
accumulate inside the cell(15) as these crystals are impermeable to the cell membranes and therefore it accumulates in healthy cells(16.17)

MTT assay used to studies the cytotoxicity of the adhesives by products (extracts) eluted from the 2% fluorinated graphene nanoparticales incorporated adhesives in comparison to the control groups on the isolated fibroblast-like cells, the incorporated and non – incorporated universal adhesive were regarded as cytocompatibility material with no significant difference(17). Also MTT assay used to studies the cytotoxicity of denture acrylic resin after successive cycles of daily overnight immersion in 1% sodium hypochlorite and 2% chlorhexidine digluconate , the result showed that residues of 2% chlorhexidine digluconate were severely cytotoxic to human gingival fibroblast compared to those of 1% sodium hypochlorite and acrylic resin (not submitted to denture cleansers), which were slightly cytotoxic(18).

In this research ALF_3 compound was studied by cyclic voltammetry to finding the oxidation–reduction properties in artificial saliva also to determine the cytotoxicity effect of AlF3 when incorporated with resin acrylic material

Materials and Methods

Artificial saliva (AS) was prepared by scientific lab of Shafeeq comp (Iraq), ALF3 was received from Reagent World, Inc. 2048 E. Francis St. Ontario, CA 91761 USA., 0.1M of HCl, 0.1M of NaOH used as buffer solution, and deionized water was used in all experiments.

Methods of Cyclic voltammetry

The cell of cyclic voltammetry (CV) cell technique was used by added of 10ml of artificial saliva in these cell and immerse the glassy carbon electrode (GCE) as working electrode, Ag/AgCl as reference electrode and platinum wire as counter electrode as shown in Fig. 1.

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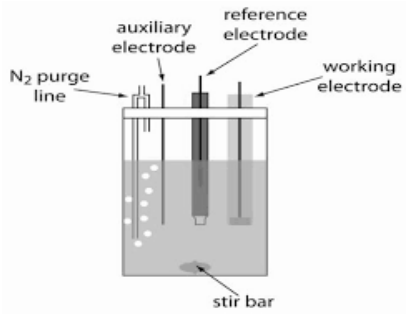

Fig. 1: Cyclic voltammetric cell

Then the three electrodes was connected with potentiostat (potentiostat/glvanostat) NuVant System EZstat (U.S.A), to calculation of the results by the cyclic voltammogram appeared in the screen of computer [1,13] as shown in Fig.2.

Fig. 2: Cyclic voltammetry set up

Cytotoxic Test

Cytotoxic assays were used as initial screening tests to evaluate the cytotoxic effect of the fluoride salt. The cytotoxicity of the different concentration of fluoride salts (,AlF3):,2%AlF3 and 5% AlF3 that were incorporated in to acrylic denture base materials were tested by MTT assay on human gingival fibroblast cell.

-Mosmann's Tetrazolium Toxicity assay (MTT assay)

Methyl Thiazol Tetrazolium (MTT) assay was conducted as an indirect screening test to determine the cytotoxic effects of the both concentration 2% and 5% of fluoride salt AlF_3 that incorporated in acrylic denture base materials were tested by MTT assay on human gingival fibroblast cell(18)

-Preparation of specimens for cytotoxicity test

Ten samples of heat cured acrylic resins were prepared from metal disc with dimensions of (10 mm in diameter and 1 mm in thickness) (18) as shown in Figure (3) by cutting metal disks into desirable shape and thickness using CNC machine. these ten samples incorporated with different concentration of different fluoride salts as follow: ,2%AlF_3 and 5%AlF_3 ,five samples for each concentration, all these specimen cultured for 24h,48h and 72h.

-Incorporation aluminum fluoride to acrylic resin material

The experimental specimens were formed by adding two different concentrations of fluoride salts (2%, and 5% by weight. %). and mixed into acrylic liquid material (monomer) relative to its different density. The weight of AL fluoride was reduced from the weight of acrylic resin material to get the accurate P/L ratio (19,20) as showed in Table 1

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Table.1. The percent of fluoride salts mixed with the heat cure acrylic resin materials (powder + liquid)

Type acrylic resin materials	Incorporation of (AlF_3) %	Amount of acrylic Powder (g)	Amount of acrylic Liquid (mL)	Quantity of AlF_3 (gm.)
Heat cure acrylic resin materials	0%	100 g	40 mL	0 mL
	2%	98 g	40 mL	2 gm.
	5%	95 g	40 mL	5 gm.

In a dry clean glass jar, the needed amount of fluoride was added completely with acrylic resin liquid materials, this mixture was mixed for 3 minutes 120 W and 60 KHz of a probe sonication apparatus gadget for fully homogeneous. Therefore, the heat cure acrylic powder materials were added quickly to the previous mixture and mixed with it according to manufacturer instructions for acrylic resin materials .Flasking ,curing ,finishing and polishing for acrylic resin specimens were done according to(21)

Fig 3: (A) Metal pattern of MTT assay test

-Preparation the culture media

For the released form, the specimen was prepared by soaking it in 5 ml of Dulbecco's Buffered Eagle Media (DMEM)Fig(4), which was then inserted in Falcon tubes, placed on a tube holder, and kept at 37C in an incubator. (Figure 4) The specimens were submerged in DMEM for exposure times of 24 hours, 48 hours, and 72 hours in order to condition the medium.

The samples were removed after the conditioning time, and the conditioned DMEM media was filtered through a Millipore filter (0.22 m) to remove solid particles and stored at -20°C until it was utilized for the MTT assay(22,23)

-Cultivation of Test Cells and MTT assay procedure

On 96-well plates, cells were planted at a density of 5x103 cells per well.

Prior to the addition of the test eluents, the cells were cultured at 37 °C with 5% CO2 for 24 hours to allow for cell attachment and acclimatization. For the MTT assay's cytotoxicity assessment, culture media containing fluoride from the sample at each exposure time was employed. A 100 mL dose of eluate was applied to each well.

Cells that weren't treated functioned as the positive control, and cells that got 10% v/v ethanol solution as the negative control (Figure 5and 6).

According to a previously reported methodology, the uptake of the Tetrazolium salt, 3-(4, 5-dinethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT assay kit)(Fig 7), was used to assess the number of live cells (17,18)

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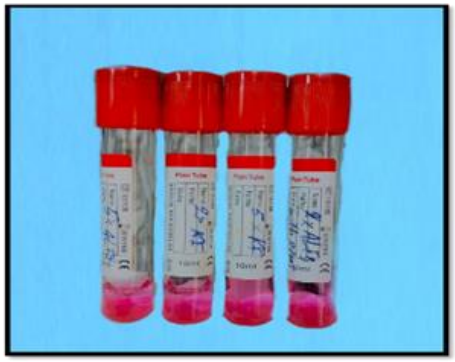

Fig.(4): Preparation of sample with Dulbecco's Buffered Eagle Media.

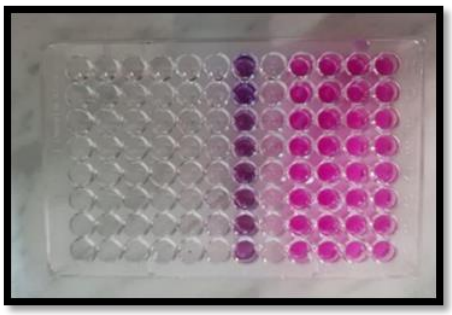

Figure (5): The 96-well plates that contained dimethyl sulfoxide (DMSO) before the incubation

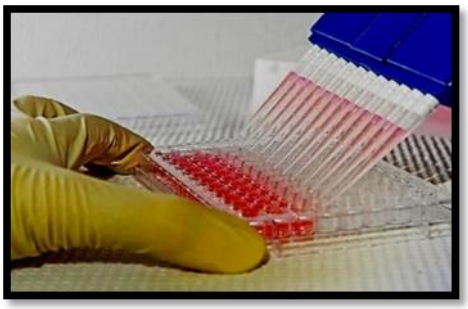

Figure (6): plated the cultured cells into the microtiter

Fig (7): MTT kit

The tested eluents were then taken out and replaced with 100 L of MTT (5 mg/mL phosphate buffered saline) for 4 hours after the plates had been incubated for 24 hours.

Following the removal of the MTT solution, 100 L of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan crystals (Figure 8).

The resulting discolouration was evaluated spectrophotometrically (ELISA reader) at 595 nm wavelength and had a direct association with the metabolic activity of cells.

The percentage of viable cells was computed as follows

% cell viability = **average** number **of viable cells** / **total number of cells**×**100**

All assays were repeated three times based on the ISO 10993-5:2009 to guarantee their reproducibility.

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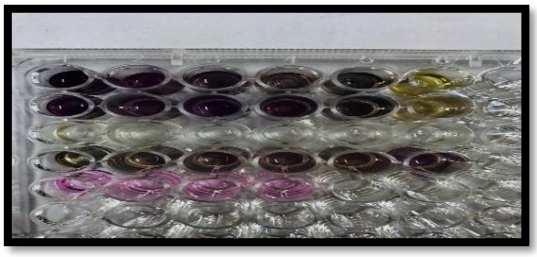

Figure (8): The 96-well plates that contained DMSO with different degree of yellow and purple colors depending on cells viability after the incubation

According to ISO 10993-12, the sample's metabolic activity decreases as the number of living cells increases.

This decline is directly related to the formation of blue-violet formazan.

According to the classification system used, the cell viability was divided into four categories: non-cytotoxic (more than 90% cell viability), slightly cytotoxic (60–90% cell viability), moderately cytotoxic (30–59% cell viability), and highly cytotoxic (less than 30% cell viability)(24,25)

Results

AlF_3 has been used in complete dentures to improve the physical and biological properties. These properties were determined by studying oxidative stress reduction in artificial saliva using a cyclic voltammeter.

-Electrochemical behavior study of fluoride salts in artificial saliva mediated by GCE using cyclic voltammetry

Figure 9 shows the electrochemical properties of each 5%AlF_3 compound in aqueous solution and artificial saliva, where the Figure shows the appearance of an oxidation peak current at potential of +1.25 V. Figure(10) showed the effect of different concentrations of 2% and 5% ALF3 the oxidation peak was enhanced by 5% AlF_3.

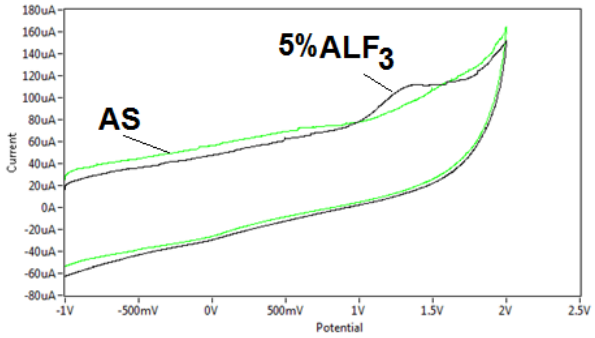

Fig. 9: voltammogram of artificial saliva only and 5%ALF_3 in artificial saliva on GCE versus Ag/AgCl at scan rate of 0.1 $Vsec^{-1}$

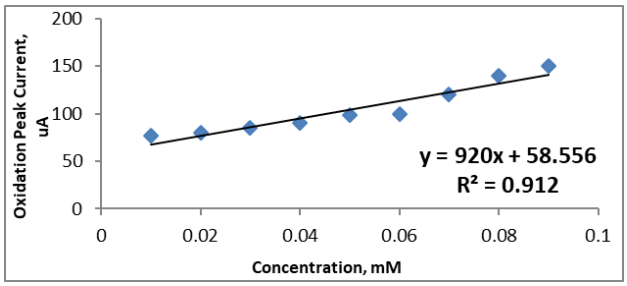

Fig. 10: Calibration curve of the oxidation peak current of 5%ALF3 in artificial saliva against to different concentrations.

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-Effect of different scan rates study of AlF3

Effect Different Scan Rates

Fig. (11) illustrated the cyclic voltammogram of ALF_3 in artificial saliva at different scan rates from 0.01 to 0.1 Vsec-1 to confirme that the cyclic voltammetric method was used in these have good results which effected on the oxidation current as in systematic enhancement as shown in Fig. (12). Oxidation equation: Y = 914.24X – 4.4286 with high sensitivity of R^2= 0.9827

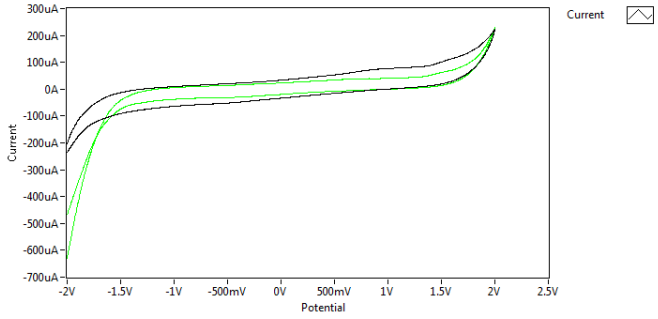

Fig. 11: cyclic voltammogram of 5%ALF_3 in artificial saliva on GCE versus Ag/AgCl at different scan rate of 0.01 - 0.1 $Vsec^{-1}$

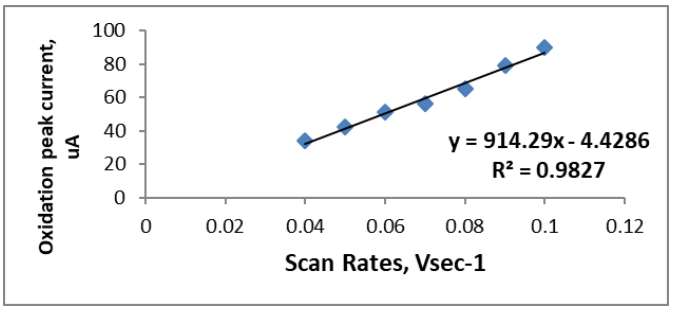

Fig. 12: Calibration curve of the oxidation peak current of 5%ALF3 in artificial saliva against to different scan rates.

It was found that ALF3 acts as an electrocatalyst for the oxidation – reduction reaction in artificial saliva. The properties of ALF3 confirm the improvement the complete denture performance in artificial saliva is observed from the calibration curve of Fig. (13), which shows the relationship between the oxidation peak current of 5% ALF_3 at different concentration (0.01 - 0.09 mM) as follows: in the equation:Y= 920X + 58.556, with high sensitivity R^2=0.912

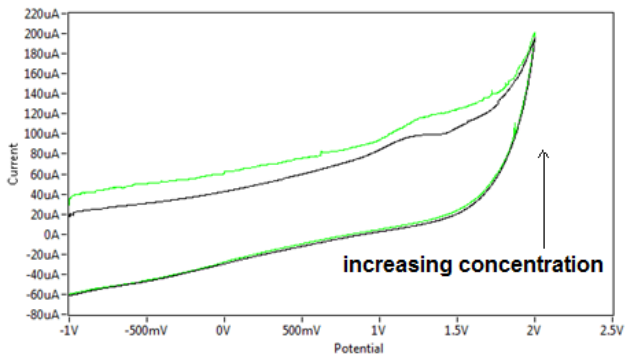

Fig. 13: voltammogram of 5%ALF_3 at different concentration in artificial saliva on GCE versus Ag/AgCl at scan rate of 0.1 $Vsec^{-1}$

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Effect Different pH

Fig. 14 shows the cyclic voltammogram of 5%ALF3 in artificial saliva at high value of pH at 11.5 (alkaline medium) and low value of 3.5 (acidic medium), the behavior of ALF_3 in acidic pH enhanced the oxidation peak current, and in alkaline medium decreased the oxidation property Fig (15)

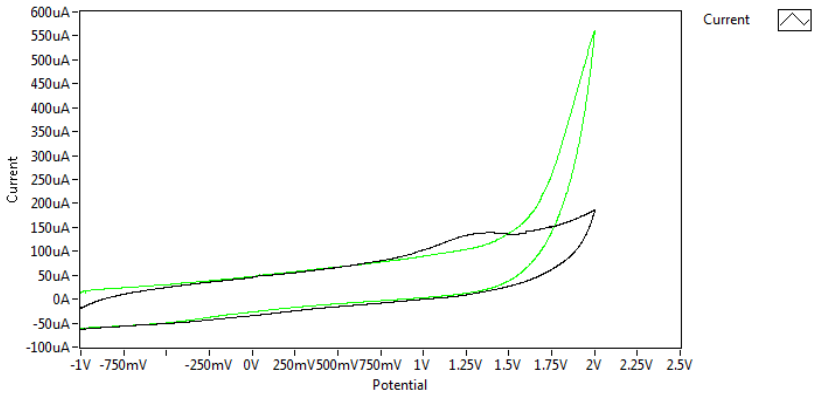

Fig. 14: voltammogram of 5%ALF_3 at different pH (green line in alkaline pH and black line in acidic pH) in artificial saliva on GCE versus Ag/AgCl at scan rate of 0.1 $Vsec^{-1}$

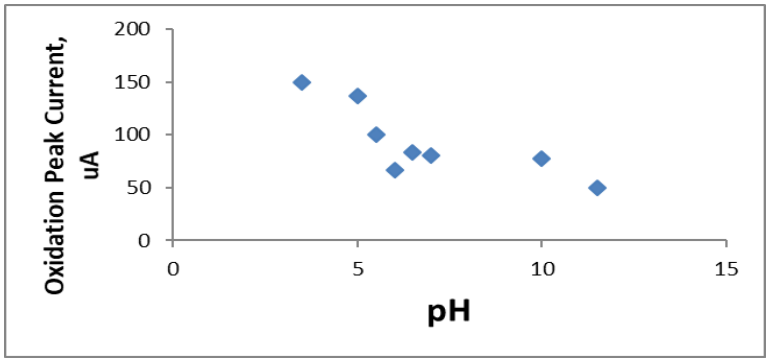

Fig. 15: relationship between oxidation peak current of 5%ALF3 in artificial saliva and different pH

-Mossman's Tetrazolium Toxicity assay(MTT) to different concentrations of fluoride salts (in vitro)

In Table (2) and Figure (16, 17 and 18) data analysis indicated that there were no statistically significant differences in cell viability of fibroblast cells mean level between two groups in time 24 and 48 hours (p-value>0.05) but, in time of 72 hours; groups of 2% salt concentrations revealed significant decrease in cell viability percentage in compare to 5% fluoride salts concentrations (p-value <0.001). The Data were expressed as (mean and standard deviation).

Table (2): Descriptive statistics of cytotoxicity test

Groups	**Time** (hours)		
	24	48	72
5%AlF3	84.2±2.7	80.0±1.4	79.0±1.0
2%AlF3	82.6±0.4	77.6±1.2	71.4±0.6
t-test	p>0.05 N.S	p>0.05 N.S	P<0.001 S.

Table (2) Fig 16,17,and 18 showed the maximum mean value of cell viability percentage in 24 ,48 and72 hours was recorded by 5% AlF_3 while the minimum mean value recorded by 2% AlF_3

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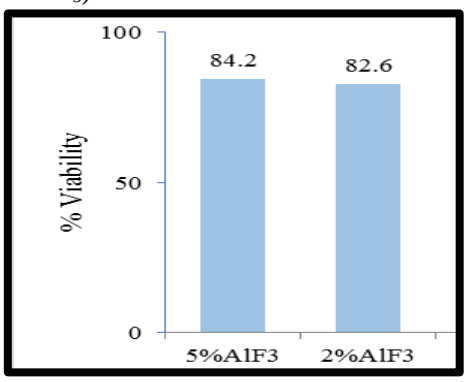
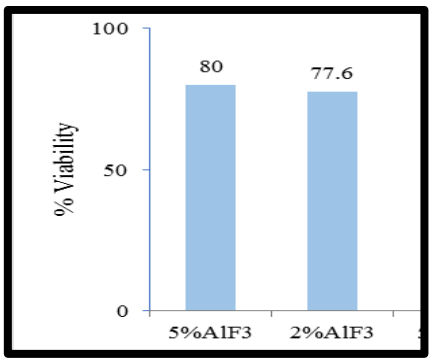
The worktop Table (2) was showed that cell viability after exposure for 24hours. was exhibited 84.2% in sample (5% AlF_3) while in the (2% AlF_3) showed 82.6% Statically, 2% and 5% AlF3 incorporated to heat cure acrylic was exhibited a non-significantly in 24 hours



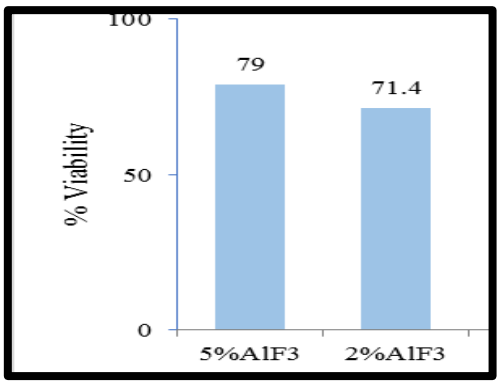
Figure(16): MTT cell viability % after 24 hours of test groups

cell viability value after 48 hours exposure period in Table (1) and Figure (17), the cell viability was 80% for 5% AlF_3 groups whereas with (2% AlF_3) group was detected 77.6% statically exhibited a non-significantly p>0.05 N.S



Figure(17): MTT cell viability % after 48 hours of all test groups

After 72hours in Figure (18), the highest cell viability was measured in the group 5%AlF_3 (79%). The group 2% AlF_3 was detected a low cell viability value (71.4) with significant difference compared with group 5%AlF_3 P<0.001



Figure(18): MTT cell viability % after 72 hours of all test groups

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Discussion

The compound of AlF_3 was used in the acrylic resin denture base to further improve the physical and biological properties. These properties were identified by means of an oxidation-reduction study in artificial saliva using cyclic voltammetry. It was found the AlF3 acts as electro-catalyst of the oxidation process in the artificial saliva, because the oxidation peak was enhansce by 5%KF and AlF3 .It was confirmed that the cyclic voltammetric method was used in the study of the AlF_3 in artificial saliva to give the good results that can be used in the research by changing the scan rates from 0.01 to 0.1 Vsec-1 and its effect on the oxidation peaks. the calibration curve of the relationship between the oxidation peak current against to the scan rates shows straight line with equation of (Radhi et al., 2017)(2). Oxidation equation: Y = 668.33X + 15.344 with high sensitivity of R2= 0.9701

The normal pH value of saliva in mouth cavity is about 6.5 [16], it's an acidic medium In this part of the research, it was studied the effect of using different pH of the medium of artificial saliva on the oxidation peak current of the 5% AlF_3 was evaluated. It showed the oxidation peaks of the Alf3 was appeared in the acidic pH (3-6) and its disappearance in the alkaline pH (7-9). So, the AlF3 in acidic medium behavior as oxidative reagent, while in alkaline pH is antioxidant (26).

The present of the KF in high concentration affects negatively in the mouth cavity, as it leads to cell damage, especially in the acidic medium (27).

Specific materials when used in the manufacture of denture care enhance the elimination of micro-organisms to promote oral hygiene(28), also some plants extracts were used for same purpose (29) ,fluoride was widely used for caries controlled in same way to improve the properties of acrylic resin (30). Controlling oral hygiene is very important especially for dental caries the pathological factor for which is oral bacteria (31).Fluoride containing dental acrylic resin demonstrated superior effectiveness to promote proper denture hygiene (32), particularly for elderly persons requiring nursing care or who have a decreased ability to perform normal activities of daily living and children who wear acrylic appliance (33,34). Fluoride containing dental acrylic resin material can improve properties of acrylic resin (35,36,37,38)

For Cytotoxicity test the in vitro study evaluated the toxicity of (2% and 5% of fluoride salt) that incorporation in to heat cure acrylic resin. Many experiments were carried out with cells in the culture rather than using the animal models. This is particularly so with regard to the determination of safety and cytotoxicity of several compounds, most studies have employed cell lines to evaluate the cytotoxicity of dental adhesives (36,37)

The MTT assay is the more accurate and most common employed for the detection of cytotoxicity or cell viability following exposure to toxic substances (17,37) Therefore, this assay is incredibly a useful tool in determining materials cytotoxicity or differentiating between multiple cell lines as it produces significant data in a short period of time, has high sensitivity and simplicity reproducibility and low costs speed (38)

There are many different in vitro studies that may be used for this purpose. MTT was a well-known and accepted cytotoxicity assay .Viable cells reduce the MTT tetrazolium salt to a blue and insoluble product formazan, which precipitates in the cytoplasm. The advantages of this assay are accuracy, speed and no need to use radioisotope. (39)

Primary gingival fibroblast cultures are more closely related to the original tissue and therefore much easier to identify (40). In addition, cultures with primary cells generally are recommended for long-term experiments as they provide heterogeneous cells with physiological states of aging and offer a metabolic state relative to their original tissue that is nearly identical (41)

Previous in vitro experiments that utilized primary gingival fibroblast cultures simulated in vivo conditions efficiently (42).For these reasons, primary gingival fibroblast cultures have been preferred to continuous cell lines.

The current study showed all of the percentage of two fluoride salts presented accepted percentage of viable cells (84.2 %). Minimum mean value of exposure time showed a slightly decrease in the cell viability from 24hours (88.6%) to 48h (77.6%) and a slight decrease at 72hours (71.4%) this was for both concentration of AlF3

Number of fibroblast cell decrease might due to low toxicity of fluoride compound that have effected viable cell and this will decrease during fluoride release, However this result were still with the normal range of viable cell of fibroblast that acceptable in MTT assay which recorded that <80% was in normal range that was accepted , there were no previous studies concerning incorporation of these fluoride salts into acrylic material order to compare MTT result with them.

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Conclusion

In the high concentration of 5%ALF3 has been affected in oxidation behavior more than low concentration of 2%ALF3. In the acidic pH of the artificial saliva has high intensity of oxidative property which act damage to the cell of mouth while in the alkaline pH of artificial saliva act as antioxidant behavior that can be used in safety. The addition of 2% and 5% of ALF3 (fluoride salts) into heat cured acrylic resin materials had no toxic effect.

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