IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 17, Issue 6 Ver. 9 (June. 2018), PP 16-26
www.iosrjournals.org

Is Incorporation of Aloe Vera Encapsulated By Chitosan Nano-Spheres To Compomer A Valid Antibacterial Approach? An In-Vitro Study

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Abstract: *Introduction: This study investigated the effect of incorporation of Aloe Vera (AV) as a filler to compomer on its antibacterial activity, solubility and surface roughness.*
Method: Compomer (Dyract XP), the control; Group 1, was modified by adding 15wt% of experimentally-prepared AV encapsulated by chitosan nano-spheres; Group 2. Discs (15x1mm) of each group were prepared and characterized. For each test, 21 discs/group were investigated. The antibacterial activity against Streptococcus mutans (ATTCC 25175) was evaluated by agar diffusion test by measuring the inhibition zone on inoculated discs. Solubility and surface roughness were investigated on discs stored in artificial saliva at 37°C with five-minute lactic acid (pH=5.2) cycling three times daily, after one week and one, three and six months. Results were statistically significant at p≤0.05.
Results: Group 2 had higher antibacterial activity (2.21±0.21 mm) than group 1(0.05±0.22 mm). The only significant change in solubility between the groups was at (0-7) days where group 1(0.390±0.016 µg/mm^3) had higher solubility than group 2 (0.158±0.039 µg/mm^3). Solubility within groups took place over a longer time for group 1; 90 days (0.690±0.022 µg/mm^3) compared to group 2; 30 days (0.577±0.044 µg/mm^3). The mean surface roughness values revealed that the baseline roughness of group 1(0.2572±0.0017µm) was lower than group 2 (0.2587±0.0020µm). This was reversed at 7 and 30 days.
Conclusion: Incorporating 15wt% freeze-dried AV encapsulated by chitosan nano-spheres to compomer imparted an antibacterial effect against Streptococcus mutans. The change in solubility and surface roughness lied within the values accepted by ISO standards for resin-based restorative materials.
Keywords: *Antibacterial activity, Chitosan, Compomer, Natural polymers and Solubility of compomer*

Date of Submission: 30-05-2018 Date of acceptance: 16-06-2018

I. Introduction

Secondary caries is considered the most common reason for long-term restoration failure and replacement. Although fluoride-containing restorative materials release fluoride ions and allow tooth remineralization, yet their antimicrobial effect is limited and inadequate to prevent secondary caries development[(1)].

Poly-acid modified glass ionomer (compomer) was developed to combine properties of both resin composite and glass ionomer[(2)]. Compomer has the advantages of superior esthetics, absence of moisture contamination, and fewer placement steps. It is accepted as having the highest level of user friendliness among all esthetic restorative materials. Compomer is also characterized by ease of polishing, less susceptibility to dehydration and radiopacity[(2)]. Despite this, compomer lacks anti-cariogenic property due to its lack of antibacterial property. To our knowledge, no research has been published to-date regarding the addition of antibacterial agents to compomer.

Several antibacterial agents as cetrimide and chlorhexidine have been reported in literature as potential antibacterial additives. However, natural phytochemicals such as, propolis, green tea, chitosan and Aloe Vera are considered better antibacterial alternatives compared to synthetic drugs[(3)]. Aloe Vera has been used for centuries in medical products for health, beauty and skin care[(4,5)]. It was reported an effective antibacterial agent against Streptococcus pyogenes, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans. In addition, it was also effective against acedogenic bacteria such as Streptococcus mutans, Lactobacillus and Actinomyces; the main contributors for caries and periodontal disease[(6)].

In the dental field, Aloe Vera was used as an additive in dentifrice[(7)], a mouth wash[(8)], a sub-gingival medication in the treatment of periodontal pockets[(9,10)], a treatment of oral lesions such as herpetic viral lesions,

aphthous ulcers etc.[11,12], an intra-canal medication[13], a storage media for avulsed teeth[14], and in prevention of halitosis and healing of extraction sockets[15,16].

Despite the numerous medical applications of Aloe Vera, its solubility remains a double weapon. Although it may be useful in cosmetics; yet this may pose a limitation if Aloe Vera were to be considered as an antibacterial filler in a restorative material. Therefore, the need to encapsulate it with a less soluble coat may be of value to allow sustained release[6]. Polymers such as chitosan have been used as vehicles in drug delivery systems[17]. Chitosan is a biocompatible, biodegradable water-soluble pH-sensitive polysaccharide[18–20]. It possesses antimicrobial activity, chemical and thermal stability and low immunogenicity. Its unique physical, chemical and biological properties; mainly its cationic character and solubility in acidic media, made it an attractive material for the use as drug carrier in drug delivery systems. Thus, chitosan could be used to encapsulate Aloe Vera to overcome its solubility.

Therefore, the aim of the present study is to investigate the effect of incorporation Aloe Vera encapsulated by chitosan nano-spheres to compomer on its antibacterial property, solubility and surface roughness. The null hypothesis was that the antibacterial property of compomer filled with Aloe Vera encapsulated by chitosan nano-spheres will be the same as conventional compomer.

II. Materials And Methods

The materials used in this study were Aloe Vera gel juice freeze-dried powder (High Altitude Organics Naturals Essentials Herbs TM, USA, B006TNERIS), Chitosan/ Poly (D-glucosamine) medium molecular weight (Sigma Aldrich, USA, MKBH1108V) and Compomer; Dyract XP (DENTSPLY, Germany, 1506000987). The composition of Dyract XP is listed in "Table 1."[21]

Table 1. The composition of compomer; Dyract XP

Product name	Type	Fillers	Filler volume (%)	Monomers
Dyract XP	Compomer	-Strontium-alumino-sodium-fluoro-phosphor-silicate glass (mean filler size 0.8µm) - Highly-dispersed silicon dioxide -Strontium fluoride	47	-Urethane dimethacrylate (UDMA) -Carboxylic acid modified dimethacrylate (TCB resin) -Triethyleneglycol dimethacrylate (TEGDMA)

1.1 Preparation and chemical characterization of the freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres" powder

Chitosan nano-spheres were prepared by the "Ion gelation method"[22]. A schematic representation of steps for powder preparation is shown in "Fig.1". All the reagents used in the powder preparation were purchased from Sigma Aldrich, USA. Preparation was carried out by the addition of 1 ml glacial acetic acid to 99 ml distilled water. Then, 0.2 g of chitosan powder were added to the acetic acid and water in a flask and left on a magnetic stirrer overnight. A weight of 0.3 g Aloe Vera powder and 0.1 g tripolyphosphate (TPP) were separately dissolved in distilled water and were added to the Chitosan / acetic acid solution while on the stirrer in a drop-wise manner successively. Finally, the solution was stored in the refrigerator at 4 ˚C for 1-2 hours before freeze-drying. The freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres" powder was prepared via grinding by a quartz mortar and pestle[23]. Chemical characterization was performed for the as-received Aloe Vera and chitosan powder (n=1) and for the prepared freeze-dried powder (n=3). The discs were analyzed by a Fourier transform infrared spectrometer (Jasco, FT/IR-6100, Japan) using absorbance mode[24]. Encapsulation of the Aloe Vera particles by the chitosan nano-spheres and powder particle size were evaluated by TEM (JEM-1400, JEOL Ltd., Japan).

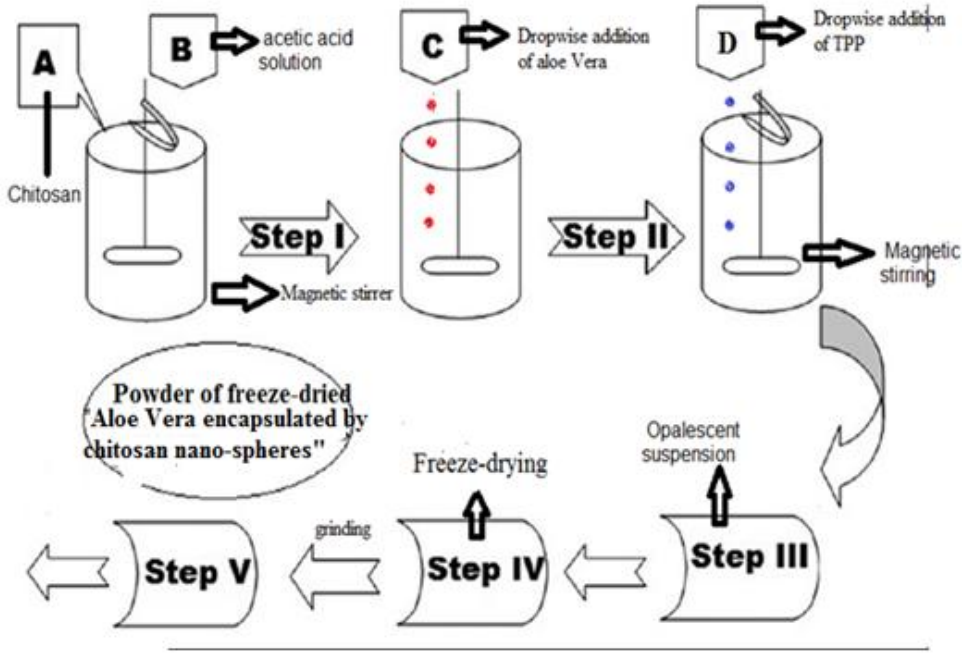

Figure1. A schematic representation of steps for freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres" powder preparation.

1.2 Sample size calculation

According to the sample size calculation by G power, with reference to the study by Tarasingh P. et al.(2015)[25], a total sample size of 42 (21 in each group) was calculated to be sufficient to detect a large effect size (0.4), with a power of 80%, and a significance level of 5%.

The samples were divided into two groups according to composition:

Group 1: Discs of commercially available compomer

Group 2: Prepared discs of compomer filled with 15 wt% freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres".

1.3 Preparation and chemical characterization of the test groups

For preparation of group 1 specimens, compomer paste (Dyract XP) was dispensed into a Teflon mold (15 ± 1 mm in diameter and 1 ±0.1 mm thick). For group 2 specimens, compomer paste was manually mixed with 0.075 g of the ground freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres" powder previously prepared. This corresponds to 15 wt% of the total weight of compomer disc. For both groups, the paste was condensed, and was packed under (4Kg) weight for standardization. All discs were covered by mylar strips and were light-cured by light-emitting diode (LED) curing unit with light intensity of 650-850 mW/Cm^2 for 20 seconds (for each side), at a tip distance of 2 mm from the sample and with 90˚ angulation. The discs were chemically analyzed by FTIR to calculate the degree of conversion[26,27] (n=3) and to characterize the prepared disc after addition of the filler. Scanning electron microscopy (SEM) (Quanta 250 FEG SEM, FEI Company, Netherlands.) was used to evaluate the filler particle distribution within the compomer disc (group 2).

1.4 Evaluation of the test groups

1.4.1 Antibacterial activity

The antibacterial activity of both groups was tested by means of the "Agar diffusion test". In order to prepare the inoculum, standardized Streptococcus mutans strain (ATCC 25175) was cultured overnight on Trypticase Soya agar (TSA) slants at 37 ˚C. Blood agar petri plates were filled with prepared blood agar infusion and were pre-incubated at 37 ˚C to check for contamination. The 24-hours inoculum of Streptococcus mutans was uniformly spread on the surface of the blood agar plates by means of a sterile swab. A total of 42 discs (21/group) were placed onto the agar medium inoculated with the streptococcus mutans in petri plates using a sterile tweezer. After 24 hours incubation at 37 ºC and pH 5 under aerobic conditions, the diameter of the inhibition zones in millimeters (mm) around the discs was measured[25,28,29].

1.4.2 Solubility test

Solubility of both groups (n= 21/group) was investigated at time intervals one week and one, three and six months. The prepared discs were dried in a desiccator at 37 ˚C for 24 hours. After 24 hours, the discs were weighed using an electrical analytical balance to obtain the initial weight of the discs **(M_1).** The discs were removed daily from the artificial saliva, rinsed thoroughly in distilled water and placed in buffered lactic acid for five minutes, three times daily. At each time interval, the discs were removed from the artificial saliva, cleaned, blot-dried and placed in the desiccator for 24 hours. Following each immersion interval, the substance loss of each specimen was measured by means of an electrical analytical balance in order to calculate the difference in weight before and after immersion **(M_3)**[30–35].

The solubility (W_{sl}) in μg/mm^3 was calculated according to the equation proposed by the ISO 4049:

$$W_{sl} = \frac{M_1 - M_3}{V}$$

Where:

M_1 is the mass of the specimen in μg, before immersion in storage media

M_3 is the mass of specimen in μg, after immersion and desiccation

V is the volume of specimen in mm^3 before immersion ($V = \Pi.R^2.h$)

2.4.3 Evaluation of surface roughness

Following each solubility interval, the surface roughness (Ra) of the specimens was recorded by means of a profilometer. Before immersion, the baseline roughness of the specimens **(Ra_i)** was measured for each disc as an average surface roughness in microns (μm) at three different points. After weighing the discs at each interval of solubility, the discs were transferred to the profilometer. Three readings at different points on the specimen surface were recorded. Data was transferred to the computer software (Elcomaster 2, Elcometer Instruments) for analysis. The mean surface roughness values (**Ra_f)** of the three readings was calculated for each disc in microns[32,33,36,37].

1.5 Statistical Analysis

Results were expressed as mean and standard deviation for each group in each test. Data was explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. One-way ANOVA was used to compare the antibacterial activity between the two groups. A repeated measure ANOVA was used to compare the solubility and surface roughness of the two groups through different time periods. Independent sample t-test was used to compare the change in solubility between the two groups in nonrelated samples. Pearson test was done to correlate between the solubility and surface roughness. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

III. Results

1.6 Results of characterization of the prepared powder and prepared discs

Characterization of the freeze-dried powder by means of Fourier transform infrared spectroscopy (FTIR) revealed that the peaks for both Aloe Vera and chitosan were observed with no absent or shifted bands; "Fig.2" and "Fig.3". FTIR spectra revealed that there was no statistically significant difference (p=0.321) in the degree of conversion between the filled and the unfilled discs (44.58% ± 5.90) and (48.49% ± 4.18) respectively. TEM micrographs showed uniform spherical particles, in the range of 114-890 nm, of chitosan around Aloe Vera; "Fig.4". Homogenously-distributed filler particles within compomer and little degree of agglomeration were observed by scanning electron microscope micrographs; "Fig.5".

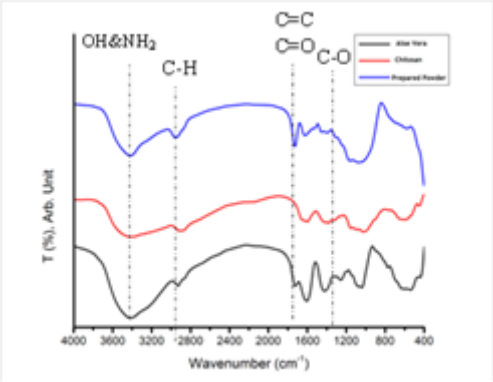

Figure 2. FTIR spectra of the as received Aloe Vera, chitosan and the prepared powder.

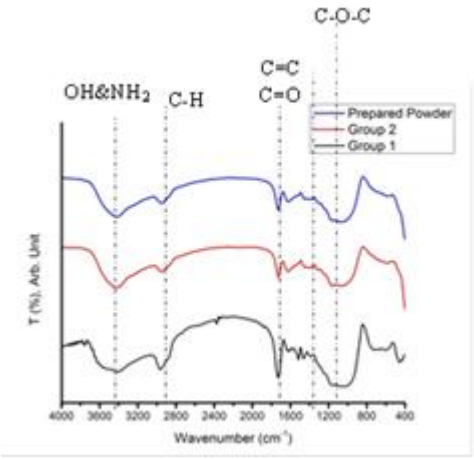

Figure 3. FTIR spectra of the prepared discs (group 1) and (group 2).

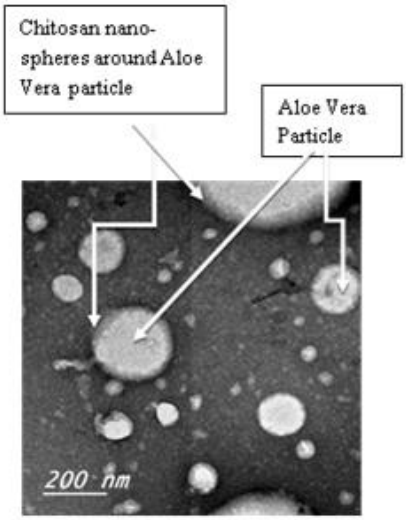

Figure 4. TEM micrographs of the prepared powder, where the areas of high electron density represent Aloe Vera and areas with lower electron density represent chitosan

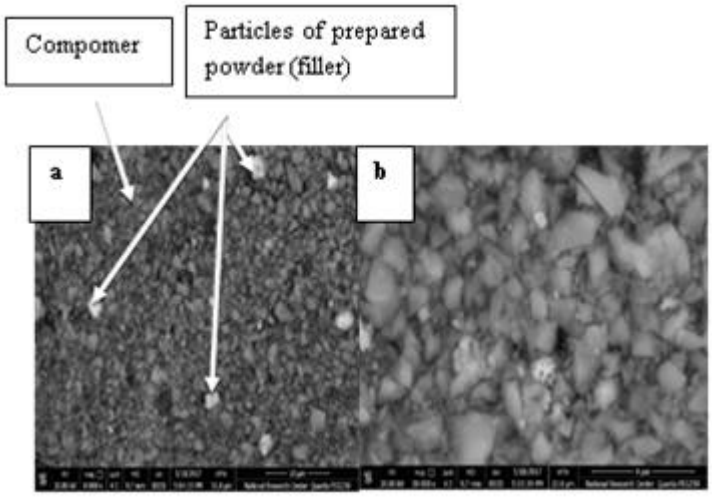

Figure 5. SEM micrographs of prepared disc

3.2 Results of the Agar diffusion test

The inhibition zones around the discs in the blood agar petri plates inoculated with Streptococcus mutans revealed that group 2 (Prepared discs of compomer filled with 15 wt% freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres") had higher statistically significant antibacterial activity compared to group 1 (compomer discs); "Fig.6", "Table 2".

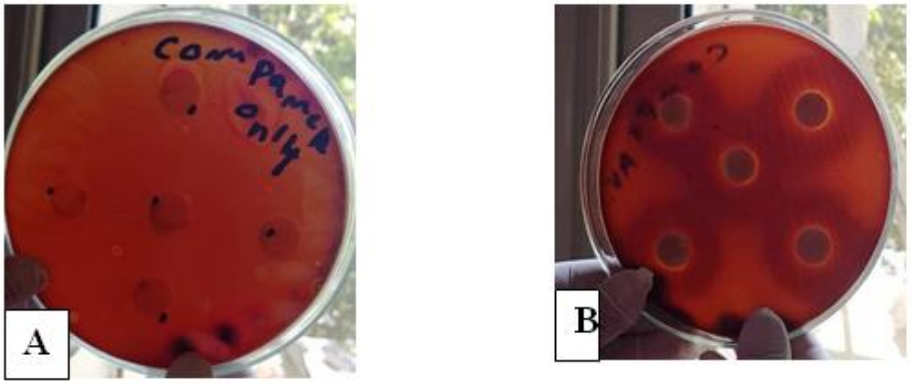

Figure 6. Inhibition zones around the prepared discs

Table 2. The mean, standard deviation (SD) values of inhibition zone (mm) in all groups.

Variables	**Antibacterial property**	
	Inhibition zone (mm)	
	Mean	**SD**
Group 1 (compomer discs)	0.05^{a}	± 0.22
Group 2 (compomer + filler discs)	2.21^{b}	± 0.21
p-value	**<0.001***	

Superscripts with different small letters indicate statistically significance difference within the same column.
*; significant ($p \leq 0.05$) ns; non-significant ($p > 0.05$)

3.3 Results of solubility and surface roughness

Repeated measure ANOVA revealed that, between the two groups, the only statistically significant change in solubility (W_{sl}) overtime took place from (0-7) days ($p<0.001$). Group 1 (0.390 ± 0.016 µg/mm^3) revealed a higher change in solubility compared to group 2 (0.158 ± 0.039 µg/mm^3). Results revealed that a significant change in solubility for group 1 specimens took place over 90 days ($p< 0.001$). For group 2, such significant change was only evident within the first 30 days ($p< 0.001$); "Fig.7".

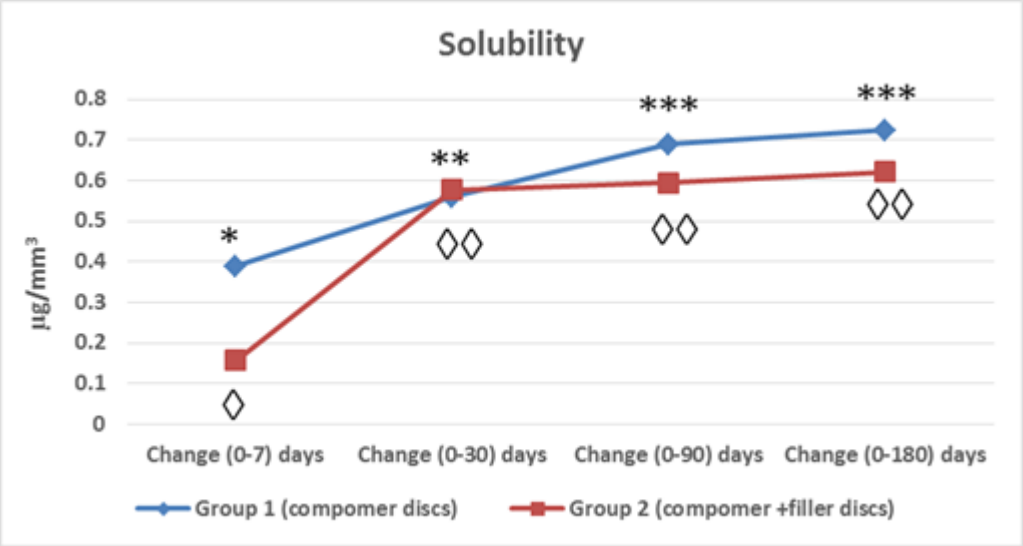

Figure7. Line diagram representing means of solubility change (µg/mm^3) over time of the two groups, * p≤ 0.05 for group 1, ◊ p≤ 0.05 for group 2. Different symbols indicate statistically significant difference between different time intervals and same symbols indicate statistically non-significant difference.

3.4 Results of surface roughness

The mean surface roughness (Ra_f) recorded after the different time intervals within each group revealed that the base line roughness of group 1 (0.2572±0.0017 µm) was lower than that of group 2 (0.2587±0.0020 µm). On the contrary, the roughness at 7 and 30 days was statistically significantly lower in group 2 (0.2534±0.0021 and 0.2522±0.0018 µm) compared to group 1 and (0.2570±0.0025 and 0.2546±0.0024 µm). A plateau in surface roughness was then noticed from 1 to 7 days and from 30 to 180 days in group 1, and after 7 days till 180 days in group 2; "Fig.8".

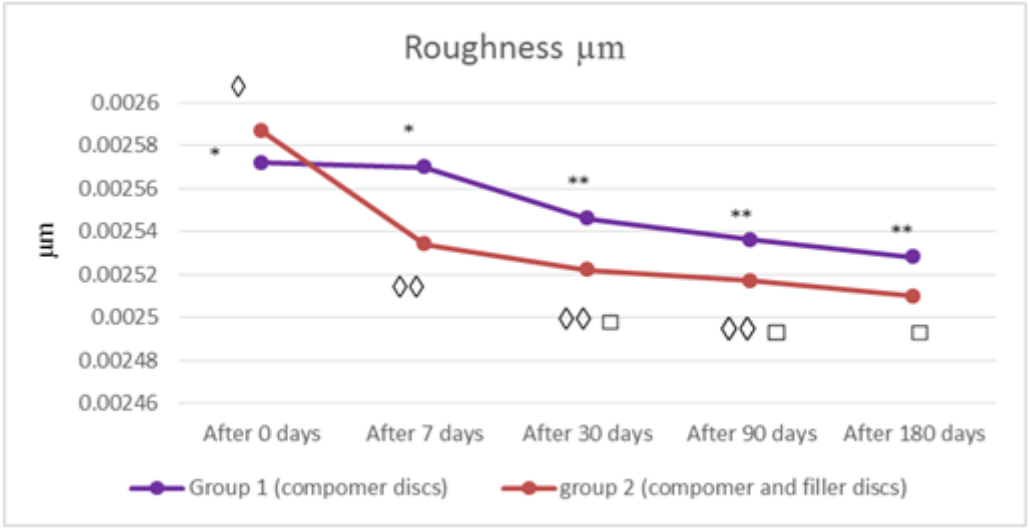

Figure8. Line diagram representing means of roughness (µm) of the two groups over time, * p≤ 0.05 for group 1, ◊ p≤ 0.05 and □ p≤ 0.05 for group 2. Different figures indicate statistically significant difference between the different time intervals and same figures indicate statistically non-significant difference.

The correlation between solubility and surface roughness is shown in "Fig.9". The pearson correlation coefficient revealed a positive relationship between solubility and roughness, r = 0.315.

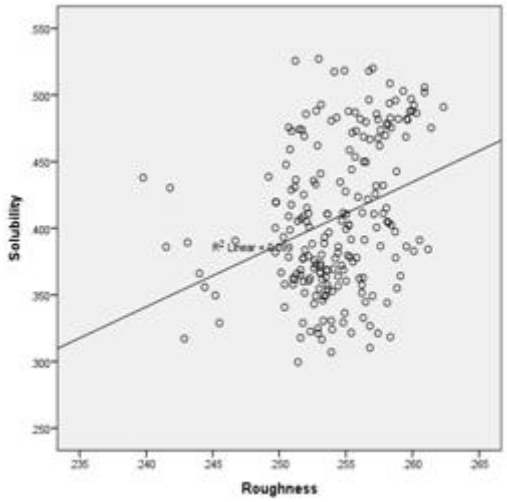

Figure 9. Scatter plot between solubility and roughness showing the best line fit where R^2 linear = 0.09

IV. Discussion

Despite the advantages of compomer as a pediatric restoration, yet they lack anticariogenic property. The aim of the current study was to evaluate the effect of incorporation of Aloe Vera encapsulated by chitosan nano-spheres as a natural phytochemical on the antibacterial properties of compomer[(2)].

The "Aloe Vera encapsulated by chitosan nano-spheres" powder was prepared by means of the "Ion gelation" method. This is a simple method which does not rely on the use of harmful organic solvent and retains the bioactivity of macromolecules during preparation[(38–40)]. Characterization of the prepared freeze-dried chitosan nano-spheres encapsulating Aloe Vera, was performed by means of Fourier transform infrared spectroscopy (FTIR) and Transmission electron microscope (TEM). FTIR is routinely used for identification of organic compounds owing to its simplicity, ease of maintenance, speed of data collection and the ease of data interpretation and reproducibility[(41)]. The presence of peaks "Fig.2,3" for both Aloe Vera and chitosan with no absent or shifted bands indicated that encapsulation of Aloe Vera by chitosan took place rather than a chemical reaction between both. The uniform spherical particles of chitosan around Aloe Vera; as revealed by the difference in electron density "Fig.4", may further confirm the formation of chitosan nano-spheres which encapsulated the Aloe Vera particles. The particle size of the prepared powder (114 to 890 nm), lied within the range normally reported for ion gelation method[(40)].

Following the powder preparation and characterization, 15 wt% of the prepared powder was added as a filler to compomer to prepare group 2 discs. Such percentage; was found suitable for obtaining a homogenous, non-friable mix. Moreover, this wt% was higher than the minimum inhibitory concentration (MIC) of Aloe Vera against Streptococcus mutans (12.5 µg/ml)[(42)]. Such higher value was proposed to ensure the effectiveness of Aloe Vera. This is because the Aloe Vera used in the current study was not in the gel form, and was coated by chitosan, and thus would not be in direct contact with the bacteria.

The prepared discs were characterized by FTIR and scanning electron microscope (SEM). The presence of the compomer spectrum, as revealed by FTIR "Fig.3.", confirmed that no chemical reaction took place between the filler powder and compomer. Moreover, absence of any difference in the degree of conversion (DC) of both groups (p=0.321), may indicate that the added filler being in the nano-metric size did not interfere with the polymerization and curing of compomer[(43)]. These findings were in accordance with Jasse F. et al., (2013)[(44)] and Rastelli A. et al., (2011)[(45)], who compared the DC of a nano-filled and a microhybrid composite. They stated that there was no significant difference in the DC between the two tested materials and reported that addition of nano-fillers did not have a negative effect on the DC.

The aim of this study was to render compomer antibacterial by incorporation of freeze-dried Aloe Vera encapsulated by chitosan nano-sphere. This was evaluated by means of "Agar diffusion test". Agar diffusion test is a simple technique accepted and approved by the clinical and laboratory standard institute for bacterial and

yeast testing[46,47]. The null hypothesis of the current study was rejected since results revealed a higher antibacterial activity of group 2; as observed from the inhibition zone; "Fig.6", "Table 2". This may be attributed to the hydrolysis of the chitosan coating; being pH sensitive in acidic conditions, and the subsequent release of Aloe Vera into the media where Streptococcus mutans is present. Contact of the Aloe Vera with Streptococcus mutans may have resulted in a direct interaction with microbial membrane proteins inhibiting the adherence of bacterial cells to the tooth surface. This would hence increase the inhibition zone by Aloe Vera[48]. These findings were in accordance with Yadav et al. (2013)[49], Bhati et al. (2015)[50] who reported that Aloe Vera reduced the count of Streptococcus mutans.

Since chitosan also possessed antibacterial effect against Streptococcus mutans[51], a confirmatory "Agar diffusion" test was carried out for compomer discs filled with 6 wt% (0.030 g) chitosan only. This was done to ensure that the obtained antibacterial results were due to the action of Aloe Vera not chitosan. Results (data not shown) revealed that the inhibition zone around discs containing chitosan was lower than the inhibition zone around discs of group 2 containing Aloe Vera.

Owing to the solubility of Aloe Vera, it was necessary to evaluate the solubility of compomer following addition of the filler. The solubility test was performed in standard viscosity artificial saliva and buffered lactic acid (pH 5.2) storage media to better simulate the oral environmental conditions. Buffered lactic acid is one of the main acids produced by cariogenic bacteria[52–55]. In addition, its pH is the pH where chitosan undergoes its phase transition, changing from an insoluble to a soluble polymer. Such transformation may allow the release of the antibacterial Aloe Vera. This was repeated three times daily for five minutes each, to correspond to the 3 daily meals.

Group 1 specimens exhibited solubility over a longer time interval; up to 90 days (0.690± 0.022 $\mu g/mm^3$) compared to group 2; 30 days (0.577± 0.044 $\mu g/mm^3$); "Fig.7". This may be attributed to both the higher resin content in group 1 and also the influence of the resin matrix[56–58]. Dyract XP; the compomer used in the current study contains UDMA and TEGDMA; which are hydrophilic monomers. Al-Qaahtani et al., (2012)[31], Arregu et al., (2016)[35] and Toledano et al. (2003)[59], attributed their findings for higher solubility of compomer (F2000 and Dyract) versus compo-glass and compo-glass F to their high content of hydrophilic monomers (UDMA and TEGDMA).

Both the solubility time interval and the change in solubility were less in case of group 2 "Fig.7". This may also be attributed to its lower percent of resin. This was in agreement with Keyf et al., (2005)[60], who stated that resin materials with higher filler content exhibit lower water sorption and solubility values. Despite the solubility of Aloe Vera, the chitosan coating (as evident by TEM) may have protected the Aloe Vera from immediate solubility, thereby lowering the solubility of the filled compomer. Although both groups revealed solubility over time, yet the obtained values were lower than the values recommended by ISO standard 4049-2009; which stated that the maximum acceptable values of solubility of polymer-based restorative materials must be equal to or less than 7.5$\mu g/mm^3$ after 7 days of immersion[61].

In the current study, a higher baseline surface roughness was obtained by group 2 (compomer + filler) "Fig.8". This may be due to the addition of the filler, which may have presented an obstacle in obtaining the finish-like surface smoothness by the mylar strip. After 7 and 30 days of immersion in the storage media, such surface roughness was reversed. This may be attributed to the solubility that took place in group 2 from (0-7) days where the homogenously non-agglomerated filler particles (as observed in SEM) may have been uniformly leached out leaving a smooth surface.

In general, a correlation exists between the solubility of the restorative materials and the increase in their surface roughness[62]. Hamouda (2011)[32] and Bajwa et al. (2014)[33], stated that there was a positive correlation between surface roughness and solubility. Although, we reported a Pearson coefficient of 0.3, yet the change in surface roughness may be partly correlated with the solubility that took place. Both groups revealed solubility. However, the higher solubility of group 1 (compomer discs) may have caused its higher roughness; where the acid may have attacked the glass fillers of compomer leading to more ion release from the bulk. In group 2, the acid may have caused the phase transition of the pH-sensitive chitosan nano-spheres rather than attacking the glass filler. This may have caused solubility of chitosan and consequently Aloe Vera leached out. As solubility decreased, the surface roughness values leveled, with both groups having the same roughness. Findings of the current study were different from those reported in literature by Tahir et al., (2004)[63], who concluded that at various pH (2, 3, 4, 5 and 6), compomer revealed very low changes in surface roughness, owing to the anhydrous nature of compomer. They also attributed this to the formation of carboxylate rich surface layer which rendered compomer resistant to surface degradation. Moreover, Munack et al., (2001)[64] reported that the surface roughness of the investigated compomers did not significantly deteriorate in different intraoral conditions over 12 months period.

V. Conclusion

Based on the current findings and considering the in-vitro study limitations, it could be concluded that addition of 15 wt% freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres" imparted an antibacterial effect to compomer against Streptococcus mutans and had no effect on its degree of conversion. Also, the modified compomer showed solubility and surface roughness values which lied within the values accepted by ISO standards for resin-based restorative materials. Therefore, it may be concluded that compomer filled with 15 wt% freeze-dried "Aloe Vera encapsulated by chitosan nano-spheres" seems to be a promising pedodontic restoration. However, further investigations regarding the investigation of antibacterial property of the prepared filler against Streptococcus mutans for a longer time and loading the compomer with different percent of filler; between 10 wt% and 20 wt% seem necessary before clinical use. In depth investigation of the physical properties as ion release analysis and the mechanical properties as modulus of elasticity and transverse strength of the filled compomer and In-vivo studies should also be carried out to assess the biocompatibility and clinical performance of the filled compomer prior to attempts to use in patients.

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