CHAPTER THREE: MATERIALS & METHOD

3. Materials and Method

3.1 Specimen selection

Human premolars of young patients aged 14-20 years extracted for orthodontic purposes were collected. The teeth were stored in 0.5% chloramine for one week, and were then stored in distilled water at 4ºC. These teeth were inspected under stereomicroscope (Kyowa optical, Japan) at 10X magnification for carious lesion and defects. Teeth with visible defects such as crack lines, demineralization including white spots and hypomineralization were discarded. Thirty teeth were selected and cleaned from debris with aqueous slurry of pumice using slow speed handpiece and rubber cup. The selected teeth were stored again in distilled water at 4ºC until ready to be prepared. The teeth were not allowed to dry at any stage of the study.

3.2 Specimen preparation

Teeth were mounted on die stone as shown in Figure 3.1. For each tooth, a Class V cavity was prepared in the middle third of the buccal surface. The outline of cavity measuring 3mm mesiodistally, 2mm occlusogingivally and 2mm in depth was initially traced with a fine tip marker. Pear-shaped diamond instrument (Super Coarse, Horico, Germany) was used on a high speed handpiece to prepare the cavity with copious water-cooling. The cervical margin for each cavity was prepared 2mm from the cement-enamel junction (CEJ). A new diamond instrument was used for every five cavity preparations. The instrument was always held at right angle to the tooth surface to produce a cavosurface angle of approximately 90º. The cavity margins were finished using straight fissure bur mounted on a slow speed handpiece to remove any unsupported enamel. The depth of the cavity was confirmed using a periodontal probe. All cavity preparations were made as uniformly as possible, in relation to its dimension,

instrumentation and outline form. Cavity outline of 3mm mesiodistally and 2mm occlusogingivally was also traced with a fine tip marker on the middle third of lingual surface of each tooth, and left unprepared.

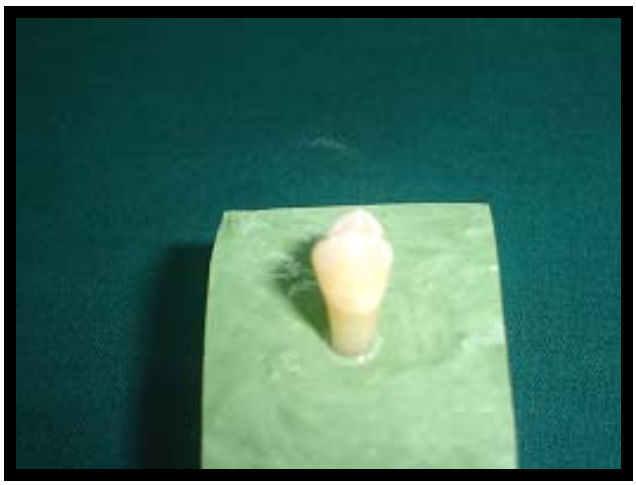

Figure 3.1 Tooth mounted on die stone.

3.3 Restoration placement

The prepared cavities were flushed and rinsed with water. It was then dried using compressed air. All thirty cavities were filled with a nanohybrid composite (Figure 3.2) shade A2 (Grandio, VOCO, Cuxhaven, Germany). The enamel and dentine were etched with 34.5% phosphoric acid gel (Vococid, VOCO, Cuxhaven, Germany) for 20 seconds, rinsed for 20 seconds and then dried using compressed air for 15 seconds. The bonding agent (Solobond M Bonding, VOCO, Cuxhaven, Germany) was applied using disposable microbrush and dispersed into a thin layer using compressed air and then cured for 20 seconds. As per manufacturer's instructions the nanohybrid composite were placed. Composite was packed into the cavity in a single increment using a plastic instrument. A clear cervical matrix strip (Dental Products Ltd, Swindow, Wiltshire, U.K) was placed on the top of the composite prior to polymerization for 40 seconds.

Composites were polymerized using a light curing unit (Spectrum800, Densply®, Caulk, U.S.A). The light emission tip of the light cure unit was held as closely as possible to the surface of composite. The light intensity of the unit was 400mW/cm^2. It was verified using radiometer which is incorporated in the light cure unit. Prepared specimens were then stored in distilled water and placed in an incubator (Memmort, Germany) at 37ºC for 24 hours. All specimens were polished sequentially using sof-lex discs (3M Dental Products, St Paul ,USA) under water spray. All specimens were again stored in distilled water and placed in an incubator at 37ºC for 24 hours prior to immersion in acid gel and qat extract.

Figure 3.2 Restorative material

3.4 Qat extract preparation

In this study, fresh qat leaves and their twigs were used to prepare the extract as shown in Figure 3.3. Fresh qat leaves was kept in aluminum foil and stored in a refrigerator at 4 ºC until it was ready to be prepared into 10% and 20% concentration.

Figure 3.3 Fresh qat leaves

10% qat extract was prepared by grinding 20g of fresh qat and twigs in 200ml deionized water. The qat extract was then stored for 4 hours in an incubator at 37ºC as described by Al-Hebshi et al., (2005). The extract were then filtered using medium filter paper size 42 (Whatman®, England) with the aid of a vacuum pump (Pump, VCP 8101, Taiwan). The pump provided additional force to obtain a pure liquid separation as shown in Figure 3.4. The pH of the extract was measured using a pH meter (HANNA Instruments, Singapore) which was earlier calibrated.

20% qat extract were prepared by grinding 20 g of qat and twigs in 100 ml deionized water. The same extraction procedure described earlier was used and the pH of qat extract was determined.

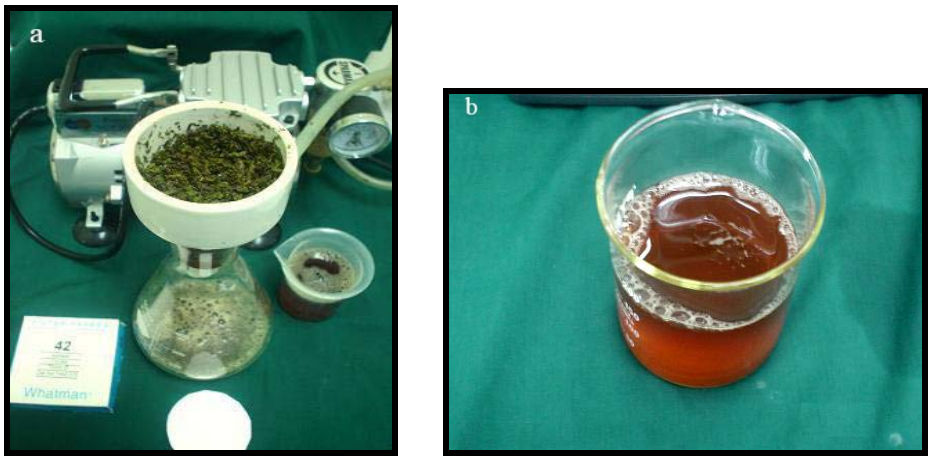

Figure 3.4 Qat extract filter procedure (a), qat extract (b)

3.5. Lactic acid gel preparations

6% by weight hydroxyethyecellulose (ALDRICH®, Germany) in 0.1M lactic acid (MERCK®-Schuchardt, Germany) was prepared as described by Tantbirojn, (2006). Acid gel pH was adjusted to match that of qat extract using 1M NaOH (HmbG Chemicals, Germany) (Figure 3.5). The pH was determined using pH meter which was earlier calibrated.

Figure 3.5 Acid gel components

3.6 Demineralization procedure

All specimens were coated with an acid-resistant commercial nail varnish (Florelle nail polish, Italy) exposing approximately 2mm of the enamel from the restoration margin on the buccal surface and cavity outline on the lingual surface. Two coats of nail varnish were applied. The first coat was left to dry for 2 hours before the second coat was applied. The specimens were then left to dry at room temperature for 24 hours before the specimens were immersed in acid gel and qat extract solution.

10 specimens were attached individually to an orthodontic wire using composite and were immersed in 200 ml of lactic acid gel (Figure 3.6) and stored in an incubator at 37ºC for four weeks. Acid gel was not changed throughout the immersion period. The pH of acid gel was monitored every two days.

The remaining 20 specimens were attached individually to an orthodontic wire using composite, and then divided into two equal groups. 10 teeth were immersed in 10% (Figure 3.6) qat extract and the another 10 teeth were immersed in 20% qat extract for four weeks and stored in the incubator at 37ºC. The qat extract was replaced with freshly prepared ones every two days.

Figure 3.6 Specimens immersed in acid gel and qat extract.

3.7 Sectioning of Specimens

After four weeks, the specimens were removed from acid gel and qat extract solution and were separated from the wire. The specimens were washed using deionized water to remove any remnants of fluid or gel. The nail varnish was removed using acetone and toothbrush. Each specimen was inspected by direct vision and under stereomicroscope (Figure 3.7) at 10x magnification to evaluate any sign of early stage demineralization prior to sectioning.

Figure 3.7 Stereomicroscope

The specimens were then embedded in clear self curing epoxy resin (Mirapox (A and B), Miracon, Malaysia) as shown in Figure 3.8. The specimens were left to set for 24 hours. The specimens were sectioned occlusogingivally through the middle of the restoration using rotating diamond blade with 0.3mm thickness attached to the Isomet low speed saw, (Buehler® Isomet low speed saw, U.S.A), under copious lubricant cooling irrigant was used as shown in Figure 3.9

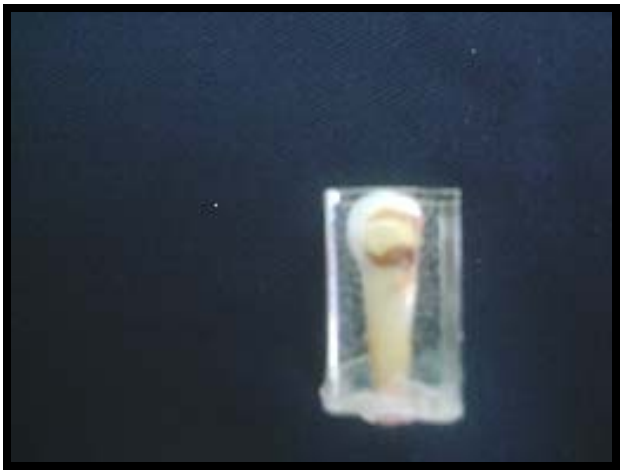

Figure 3.8 Specimen embedded in epoxy resin.

Figure 3.9 Isomet low speed saw.

Two longitudinal sections were obtained from each tooth with approximately 300µm in thickness. Each section was polished and grounded to an approximate thickness of 100 ± 20µm using 1000 grit silicon carbide grinding paper (Carbimet®, Buehler,U.S.A), mounted on a rotary grinder machine (Struers rotopol-1, Copenhagen, Denmark) at 150 rpm under copious water irrigation (Figure 3.10).

Figure 3.10 Grinding and polishing machine.

The thickness of each section was recorded using micrometer (Mitotoyo, Japan) as shown in Figure 3.11. Two sections from the same tooth were kept together in a labeled container filled with distilled water. All specimens were stored for 24 hours at room temperature prior to microscopic evaluation.

Figure 3.11 Micrometer.

3.8 Microscopic evaluation and measurements.

3.8.1 Smooth enamel surface

The lingual surface of each section was viewed under light transmitting microscope mounted with polarized glass at 20X magnification (Nikon Eclipse E400, Japan) (Figure 3.12). Surface lesion on the smooth enamel surface was recorded as the outer lesion as illustrated in Figure 3.13. The lesion was measured from the outer surface to the deepest part using Image analyzer software system (Image-Pro, Version 4.5, Media Cybernetics, L.P, Maryland, U.S.A) linked to a microscope. Five readings were taken at an interval of 0.2mm. For lesion with irregular advancing fronts, only the deepest part was recorded. All relative measurements from the two sections of each lesion were averaged and computed.

Figure 3.12 Polarized light microscope

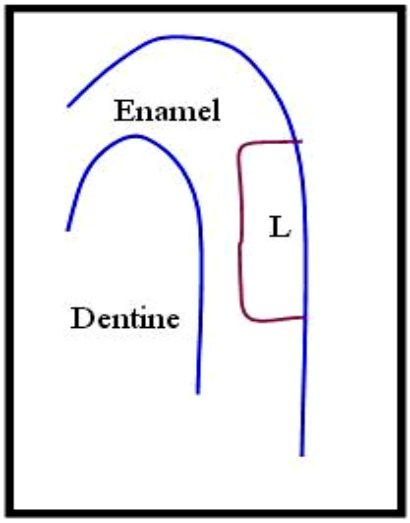

Figure 3.13 Schematic representation of smooth enamel lesion evaluation under polarized light microscopy. L: Lesion

3.8.2 Restoration interface

The buccal surface of each section was viewed under light transmitting microscope mounted with polarized glass at 20X magnification. The lesion was divided into two parts, outer lesion and wall lesion at coronal and cervical aspect of composite restoration (Figure 3.14).

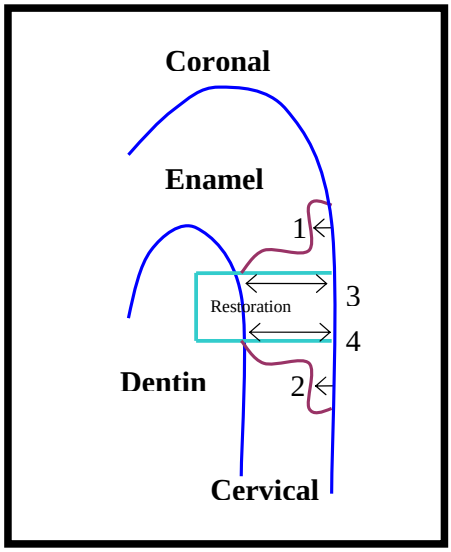

Figure 3.14 Schematic representation of lesion evaluation at restoration interface under polarized light microscope. Outer surface enamel lesion depth (1, 2), Enamel wall lesion depth (3, 4). Adapted from Nury & Alev (2002)

The outer lesion and wall lesion at restoration interface from both coronal and cervical margins were measured in each section using image analyzer software linked to microscope. For outer surface lesion five readings were taken at an interval of 0.2mm from the outer edge of the lesion towards the inner most edge of the lesion adjacent to restoration. An average reading were taken and computed. The wall lesion was measured from the cavosurface margin to the deepest point of the lesion. All measurements from the two sections of each lesion were averaged and computed.

3.9 Research design and data analysis procedures

In this experimental research, two research designs will be utilized. First, univariate between-subjects design with one independent variable (IV) and one dependent variable (DV). The three IV levels is the demineralizing agent or solution type consisting of acid gel, 10% qat extract and 20% qat extract. The DV is the extent of demineralization of the smooth enamel surface measured as enamel lesion depth. The second research design is a multivariate between-subjects design with the same IV but four different DVs; Coronal Outer Lesion Depth, Coronal Wall Lesion Depth, Cervical Outer Lesion Depth, and Cervical Wall Lesion Depth.

For the first research design, the One-way Analysis of Variance (ANOVA) will be used to examine whether there will be significant differences in the extent of demineralization of the enamel as a consequence of the different demineralizing agent i.e., acid gel, 10% qat extract, and 20% qat extract at 95% confidence level. To ensure valid statistical several assumptions underlying the one-way ANOVA that will be investigated. Firstly, DV i.e. enamel lesion depth must be normally distributed for each of the groups as defined by the different levels IV i.e. acid gel, 10% qat extract and 20% qat extract. The assumption normality can be ascertained by examining the shape of the distribution of the dependent variable as well as the presence of outliers and extreme values. Secondly, it is required that the variances of the DV are the same for all of the groups. If unequal variances are found, it is, therefore, necessary to choose a post hoc multiple comparison test that do not require that the group's variances to be equal

For the second research design, the statistical procedure to be used is the One-way Multivariate Analysis of Variance (MANOVA). This procedure is considered appropriate as more than one DV is involved in this analysis (Clark-Carter, 1997;

Kinnear & Gray, 2005). There are several assumptions underlying the one-way MANOVA:

i) The dependent variables meet the multivariate normality assumption.

ii) It requires that the population variances and covariances among the dependent variables are the same across all levels of the factor.

iii) MANOVA similar to ANOVA is sensitive to outliers and it is especially worrisome as outliers can produce either a Type I or a Type II errors.

iv) MANOVA assumes linear relationships among all pairs of DVs (Tabachnick & Fidell, 2001). Deviations from linearity reduce the power of statistical tests (Tabachnick & Fidell, 2001).

3.10 Intraexaminer Reliability

In order to ensure that reliable measurements of depth of enamel demineralization were obtained, the statistical analysis for consistency of measurements will be undertaken. In this study, two sets of measurements will be taken for all specimens in the acid gel on two different occasions at an interval of one week. Consistency of the measurements will be then estimated using the intra-class correlation coefficient.