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RESEARCH ARTICLE

IN VITRO ASSESSMENT OF ALFALFA PLANT EXTRACT IN REMINERALIZING SUBSURFACE CARIOUS LESIONS USING QUANTITATIVE ENERGY DISPERSIVE X-RAY ANALYSIS WITH SEM

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ABSTRACT

Objectives: Alfalfa (Medicago sativa), a mineral- rich medicinal herb is a powerhouse of calcium, phosphorous, magnesium, potassium, sodium and antioxidants (vitamin A, B, C, D, E, K). All these attributes appear to maintain a big potential for investigation of re-mineralization properties of this medicinal herb. The objective of this in-vitro study is to investigate the efficacy of Alfalfa plant extract in remineralizing artificially induced carious lesion and to compare it with other commercially available re-mineralization products such as the CPP-ACP and fluoride varnish using high resolution scanning electron microscopy (SEM) and Energy dispersive X-ray spectroscopy (EDX).

Materials and method: Eighty demineralized sample were divided into four test groups, each containing twenty teeth. Group A –aqueous alfalfa plant extract, Group B - CPP-ACP (Tooth mousse), Group C – fluoride varnish, Group D - control. After 30days period, the entire test groups were evaluated with HRSEM and EDAX. The obtained data were analyzed statistically using one-way ANOVA, Post hoc analysis.

Result: Statistical analysis demonstrated that group A and group B received a significantly higher amount of remineralization followed by group C.

Conclusions: Alfalfa, CPP-ACP showed marginally more remineralization than fluoride varnish. Thus, alfalfa can be considered as a substitute for CPP-ACP as a remineralizing agent.

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INTRODUCTION

Dental structure within the oral environment is constantly exposed to demineralization and re-mineralization, and if the existing equilibrium is disturbed for any reason, it will result in the destruction of tooth structure (Yamaguchi *et al*., 2006). In a neutral environment, the enamel hydroxyapatite crystals are in equilibrium with the saliva (Silverstone *et al*., 1988). Under physiological conditions, oral fluids have calcium Ca and phosphate-P in supersaturated concentrations with respect to the mineral phase of enamel, and as a result, calcium and phosphate ions are continually deposited on the enamel surface or are redeposited in enamel areas where they were lost (Gjorgievska *et al*., 2013).

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This can be considered a natural defense mechanism promoted by saliva to preserve the mineral structure of enamel in the oral cavity. A carious lesion begins when there is a drop in pH due to the formation of organic acids. At pH=5. 5 and below, hydrogen ions produced by bacterial metabolism, reacts with a phosphate group of enamel crystals(Lata *et al*., 2010). The effect of these responses is the dissolution of enamel or enamel demineralization. Induced re-mineralization occurs when the pH rises and there is deposition of calcium, phosphate, and fluoride ions in the form of fluorapatite, which is more resistant to crystal dissolution by organic acids (Margeas *et al*., 2006). Thus, the best strategy for caries management is to focus on the methods of improving the re-mineralizing process with the help of re-mineralization products (Rao and Malhotra, 2011). In the last few years, there has been an exponential growth in the field of herbal medicine and these drugs are gaining popularity both in developing and developed countries because of their natural origin and fewer side effects (Rupeshkumar *et al*., 2014).

The chemical constituents present in them are a part of the physiological function of living flora and hence they are believed to have better compatibility with the human body (Kamboj 2000). According to World Health Organization (WHO), developing countries were encouraged to utilize traditional medicine in situations that modern medicine approach does not have any offer (Pal and Shukla, 2003). Mikaili *et al*. (2011) found that over 80% of people in developing countries, applies herbal remedies for curative needs. Thus, in search of an herbal re-mineralizing agent, a mineral- rich medicinal herb Alfalfa (Medicago sativa) also called Lucerne was chosen for the study. Alfalfa comes from the Arab literature which means "The Father of All Plants" (Mikaili and Shayegh, 2011). Today, alfalfa, considered as the green food of the millennium, has dietary uses because of its good nutritive qualities and has its therapeutic uses in thetreatment of GIT disorders, enhances the immune system, prevents anemia and detoxifies the body(Furgał and Milik, 2008; Głowniak *et al*., 2007). The alfalfa plant is naturally high in many essential vitamins, including vitamin A (β-carotene), B1, B2, B3, B5, B6, B8, B9, B12, C, D, E, K, U(Bertin, 2008; Grela, 2008; Grela and Kowalczuk-Vasilev, 2010; Zanin, 2009). Each individual vitamin, has an abundance of health benefits in itself, making them crucial to overall human health. The herb gets its antifungal and antibacterial properties from the presence of saponins, which is effective against various gram-positive and gram-negative organisms. In addition to that, Avato *et al*. (2006) observed antimicrobial property of saponin from Medicago species and concluded that they show a strong antibacterial activity against gram-positive bacteria Bacillus cereus, B. subtilis, Staphylococcus aureus and Enterococcus faecalis, and control some yeast-like fungi. Not only does the alfalfa plant contain a full spectrum of important vitamins, but it is also loaded with extremely important minerals such as biotin, calcium, iron, phosphorous, magnesium, manganese, potassium, copper, zinc, silica and many others (Bertin, 2008; Grela, 2008; Grela and Kowalczuk-Vasilev, 2010; Zanin, 2009). Table 1 shows the Nutritional content of 10 g Lucerne leaf concentrate.

Table 1. Nutritional content of 10 g lucerne leaf concentrate – APEF data

Component (per 10 g)	Nutrients provided	Quantity (mg)
Proteins (4.9 – 5.3 g)	Lysine	321
	Tryptophan	100
	Threonine	239
	Cystein	59
	Methionine	112
	Valine	308
	Leucine	443
	Isoleucine	242
	Tyrosine	242
	Phenylalanine	250
Lipids (1 g) among which are :	Linolenic acid	332
	Linoleic acid	133
Minerals (0.9 – 1.3 g)	P (available)	70
	Ca	320
	K	70
	Fe	7
	Na	0.5
	Mg	13
	Mn	0.6
	Zn	0.2
	Cu	0.078
Vitamins	Beta-carotene	920 µg RE
	Vitamin E	3
	Vitamin B9	0.03
	Vitamin K	0.3
	Choline chlorhydrate	6.4

As the plant extract is rich in minerals, and insufficient literature supporting the re-mineralizing properties, the present study aimed to investigate the efficacy of the Alfalfa plant extract on the re-mineralization and to compare it with other commercially available re-mineralization agents, namely Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) tooth mousse and fluoride varnish using scanning electron microscopy with an energy dispersive X-ray analysis.

Scanning electron microscopy with an energy dispersive X-ray analysis attachment is used as a micro analytical technique to quantitatively estimate the amounts of mineral in a given tooth sample. The principle is based on the energy emitted in the form of X-ray photons when electrons from external sources collide with the atoms in a material, thus generating characteristic X-rays of that element. When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms on the specimen's surface (secondary electrons). A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted (characteristic X-rays) to balance the energy difference between the two electrons. The EDX X-ray detector measures the number of emitted X-rays vs. their energy. The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the energy vs. relative counts of the detected X-rays is obtained and evaluated for qualitative and quantitative determinations of the elements present in the specimen using a computer-based program (Hegde *et al*., 2007).

MATERIALS AND METHODS

A total of 80 premolars (maxillary and mandibular first premolars) that was indicated for orthodontic extractions were included in the study. A simple random sampling was done in the selected cases with complete root formation. Grossly carious or damaged teeth, fractured teeth were excluded from sampling. After obtaining an informed consent from the patients, extraction procedure was performed following strict sterilization protocols. The collected teeth were thoroughly cleaned of its debris, calculus and soft tissues and inspected for intact surfaces free from caries, hypoplasia and white spot lesion. The teeth were stored in 0.1% thymol to prevent any fungal or bacterial growth until further processing. Roots were sectioned at cementoenamel junction. The coronal part was then sectioned mesiodistally into 2 halves using a high-speed diamond tipped disc. The buccal halves of the teeth were used for the study, considering the ease of mounting the sample on a scanning electron microscope (SEM)

Preparation of windows

Two square pieces of plaster adhesive tape measuring 3mm length x 3mm width (mesiodistal) were placed on the occlusal one-third & cervical one-third of buccal surfaces of all samples before applying acid-resistant nail varnish; this was done to limit the area of study. The size of the window was measured using UNC 15 periodontal probe (Hu-Friedy). Each sample was dried with compressed air prior to application of nail varnish. The nail varnish was allowed to dry at room temperature for 20 minutes and the second application of nail varnish was placed

Preparation of samples for Demineralization

Following additional 20minutes of drying of nail varnish at room temperature, the plaster adhesive tapes were removed.

Artificial carious lesions were created in the exposed enamel which was uncovered by nail varnish and suspending all teeth in a demineralising solution which was a mixture of calcium chloride (2.0 mmol/L), tri sodium phosphate (2.0 mmol/L) in acetate buffer (75 mmol/L) solution at pH 4.6 for 5days (Shashikala and Sheela, 2011).

After 5 days the teeth were removed from the demineralising solution & washed. The teeth were randomly divided into four groups of 20 teeth each.

Group A– Alfalfa liquid extract. (Dr. Reckeweg-Germany Alfalfa mother tincture)

Group B- CCP-ACP (GC Tooth Mousse cream, RECALDENT TM)

Group C- Fluoride varnish (Fluor-Protector containing 0.9% difluorosilane by weight, Ivoclar Vivadent)

Group D- Artificial saliva (Demineralized sample with no treatment).
Among the two windows created on each tooth, the upper demineralized window in all specimens was covered with masking tape to serve as a baseline value of demineralization while the lower window was used for remineralization in each group.

Preparation of samples for Remineralization

The samples in each group were treated with their respective remineralising agents using disposable microtip brush for 4 minutes twice daily with 12 hours duration between subsequent applications for a period of 30 days, after which they were rinsed with deionized water, dried and stored in artificial saliva (Todd *et al*, 1999), consisting of 20 mmol/l NaHCO3, 3 mmol/l NaH 2 PO 4, and 1 mmol/l CaCl 2 at room temperature and neutral pH. This was done to stimulate oral condition. The samples in the control group were incubated in artificial saliva at 37 ° c after demineralization for a period of 30days but received no remineralization treatment.

At the end of 30 days following the removal of masking tape from the upper window, the samples were subjected to SEM-EDX study to obtain demineralization and remineralization values. The quantitative assessment of the changes in mineral profile was studied by EDX. The qualitative topographical assessment was performed by HRSEM. The samples were viewed under 500x, 1000x and 1500x magnification.

RESULTS

The present study evaluated the re-mineralization potential of Alfalfa plant extract, CPP-ACP and fluoride varnish on artificial enamel subsurface lesions using SEM-EDX. Energy dispersive X-ray analysis was used to determine calcium and phosphorus content in weight percentage of demineralized and re-mineralized enamel. Figure 1 shows, Microanalysis reports of various elements by Energy dispersive X-ray analysis of group A (alfalfa), group B (CPP-ACP), group C (fluoride varnish) and group D (control). The calcium and phosphorus content were then converted into Ca/P ratios for each group of the obtained data. The obtained data were subjected to statistical analysis using one-way ANOVA, Post hoc analysis and $P \leq 0.05$ was considered to be significant.

Intergroup comparison using one-way ANOVA, showed that there was no statistically significant difference in the mean value obtained between all 4 groups after demineralization. This shows that the demineralization solution that was used for the study produced uniform artificial carious lesions. And when the mean Ca/p ratios of the study groups after remineralization were compared, showed a significant difference (p-value < 0.001), implying that all the three re-mineralizing agents had different remineralisingpotenials (Table 2). Post Hoc analysis was done to assess the best re-mineralizing agent among the 3 re-mineralizing agents, the results showed that alfalfa and CPP-ACP were similar in remineralising properties with no stastical significant difference, followed by fluoride varnish with a significant difference when compared to other study groups. (Table 3 & 4) The HRSEM pictures revealed mineral deposits on the surface when each test group was compared with the control group (figure 2). In group A (alfalfa) and group B (CPP-ACP), enamel rods and prismatic structures are not noticeable, but the area of calcified deposits is more evident and are seen concentrated along the porous defects. In group C (F. Varnish), the area of remineralization is seen along with some areas of porosities. In group D (Control), the parasites are more evident with no areas of calcified deposits.

DISCUSSION

The re-mineralization process is a slow precipitation process of mineral constituents into hard dental tissues (Vollenweider *et al*., 2007). It has been suggested that several individual factors could have an impact on re-mineralization, including activity and the depth of the lesion, diet, salivary flow, plaque removal strategies, and use of fluoride. These factors modulate the natural process of arrest of lesion development and may shift the balance back in favor of re-mineralization (Gjorgievska 2013). The remineralization process involves diffusion of calcium and phosphate ions through the protein/water-filled pores of the carious surface enamel into the body of the enamel lesion. Once in the body of the enamel lesion, these calcium and phosphate species increase the activities of Ca2+ and PO43-, thereby increasing the degree of saturation with respect to hydroxyapatite (Reynolds 1997). Recently an increasing interest has been observed in various dietary supplements that provide specific physiological and prophylactic effect and thus, contribute to an improvement of oral health status. One of such dietary supplements is alfalfa concentrate (Zanin 1998). Alfalfa is rich in calcium, iron, copper, manganese, phosphorus, potassium, silicon, zinc and many vitamins. Great quantities of calcium and iron should also be highlighted. Calcium (32.9 g kg-1 APC concentrate on average) is indispensable for skeletal and tooth development (Balch 2000). Alfalfa is high in mineral content and, because of this, it is ideal for bones, joints and skin. It promotes both bone and teeth health (Mikaili and Shayegh, 2011). Dr. John Christopher and Kirsch further supported the mineral-rich herb alfalfa would help to rebuild tooth enamel. The University of Indiana further stated Alfalfa is especially rich in iron, calcium, and phosphorus, all necessary for strong, healthy teeth (Curezone.org…c2000-2016) The result of this in vitro study showed that alfalfa extract, re-mineralized subsurface lesion. As there are no studies conducted to assess the re-mineralization potential of alfalfa and scanty literature regarding the re-mineralization properties, the result of our study was in accordance with the various authors who stated that alfalfa has re-mineralization potential.

Table 2. Comparison of mean Ca/p ratios of demineralized, remineralized and difference between demineralized and remineralized enamel samples using one-way ANOVA

GROUPS	No. of samples	Ca/P ratio of demineralized samples (Mean)	Ca/p ratio of remineralized samples (Mean)	Ca/p ratio of difference b/w demineralized and remineralized (Mean)
GROUP I (Alfalfa)	20	1.2377	1.6165	0.3788
GROUP II (CPP-ACP)	20	1.2584	1.5804	0.3204
GROUP III (F. Varnish)	20	1.2487	1.3565	0.1078
GROUP IV (control)	20	1.2200	1.2207	0.0007
P value		0.13	<0.001	<0.001

Table 3. Intergroup comparison between the three experimental groups for remineraililzing potential

Ca/P wt% - Remineralized				
Post hoc analysis				
Groups	N	Subset for alpha = 0.05		
		1	2	3
Control	20	1.220730		
F. Varnish	20		1.356530	
CCP-ACP	20			1.580355
Alfalfa	20			1.616480
Sig.		1.000	1.000	.345

Table 4. Post hoc analysis; Intergroup comparison between the three experimental groups for Difference b/w demineralization and remineralization

Difference b/w DML and RML				
Post hoc analysis				
Groups	N	Subset for alpha = 0.05		
		1	2	3
Control	20	.000740		
F. Varnish	20		.107790	
CCP-ACP	20			.320360
Alfalfa	20			.378795
Sig.		1.000	1.000	.090

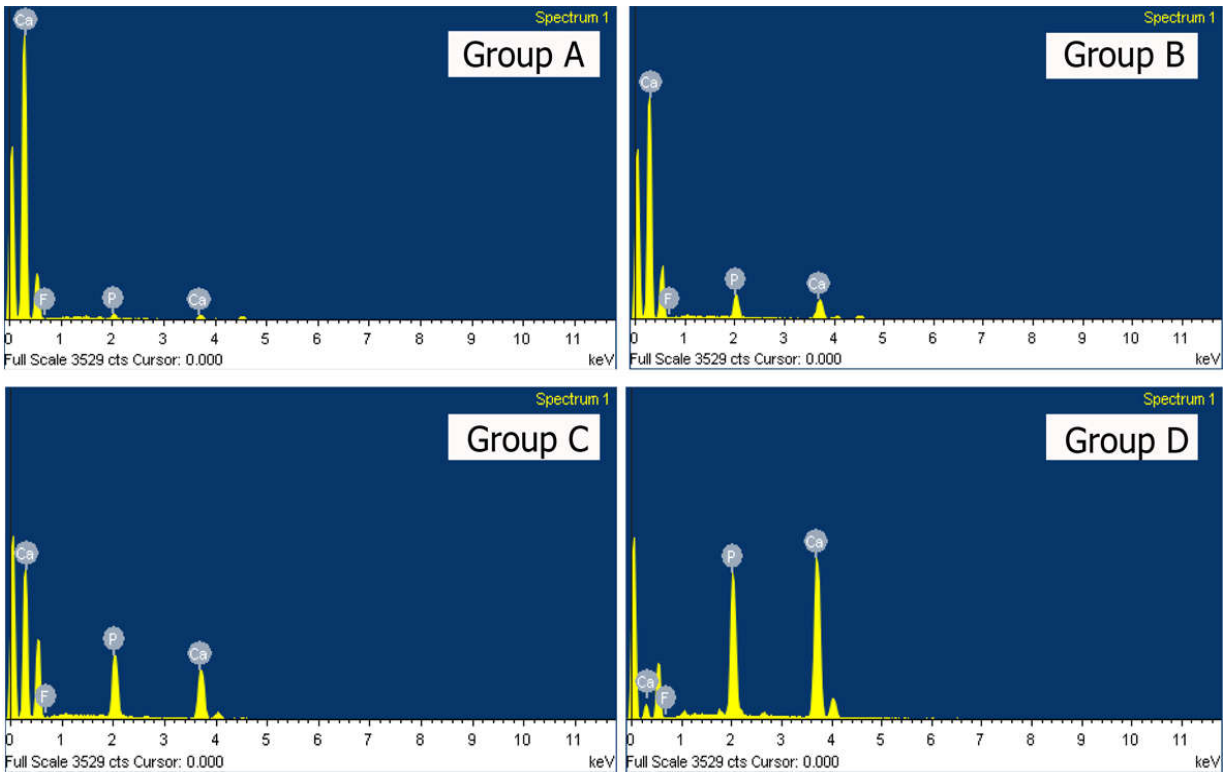

Figure 1.

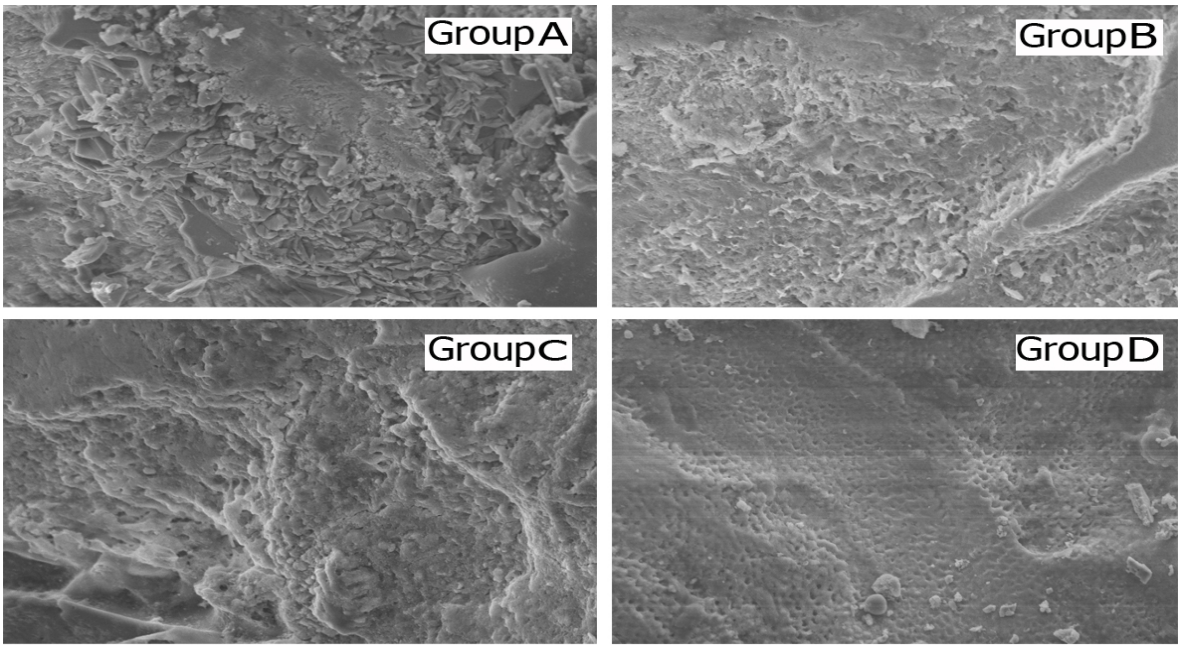

Figure 2.

The exact mechanism of action on re-mineralization potential of alfalfa is not known. Each calcium phosphate phase possesses its own thermo dynamical solubilities. For example, at pH=7 and 37ºC, HA is the most stable phase. The stability of the calcium phosphates is the characteristics of the solution in which these salts are formed or placed, namely the solution supersaturation in free calcium and phosphate ions (Tang, 2001). At a given pH and temperature, a free calcium and phosphate ion containing solution can be categorized in three different states: (i) the stable (undersaturated) zone, where crystallization is impossible, (ii) the metastable zone (supersaturated), where spontaneous crystallization of calcium phosphate salt is improbable, although the concentrations are higher than the ones corresponding to the salt solubility. If a crystal seed were placed in such a metastable solution, growth would occur in the seed; (iii) the unstable or labile (supersaturated) zone, where spontaneous crystallization of calcium phosphate is probable, but not inevitable (Mullin, 1993). Extracellular fluids that are supersaturated for calcium and phosphate may induce the nucleation and growth of new calcium phosphate crystals (Barrere 2006). This can be hypothesized for the re mineralization potential of alfalfa, as the liquid tincture is a supersaturated solution of minerals especially calcium. In the present study, EDX has been used for elemental analysis at the ultrastructural level, to measure the small changes in a tooth's mineral content. It is a microanalytical technique that is used in conjunction with SEM wherein SEM does the structural analysis and the elemental analysis is done by EDX (Hegde 2007).

Multiple studies have shown that CPP-ACP containing products prevent demineralization and promote enamel remineralization both in vitro and Invivo. The result of this in vitro study showed that 10% CPP-ACP paste remineralized subsurface lesions in human enamel. The result of this was consistent with the study which demonstrated that CPP-stabilized calcium phosphate solutions remineralized subsurface lesions in human enamel (Reynolds, 1997). The result of our study also showed that re-mineralizing potential of CPP-ACP was better than fluoride varnish.

This corroborates a study which found that use of CPP-ACP paste(63%) resulted in the greatest reduction size of the white spot lesion in millimeters followed by fluoride varnish(51%) after 12months (Memarpour, 2015). The study evaluated the effect of the fluoride gels and varnishes in comparison with CPP-ACP complex on the surface micro hardness of early enamel lesions and concluded that both Fluoride varnishes, and CPP-ACP tooth Mousse showed similar results in re-mineralizing the enamel lesion (Ambarkova, 2013) which is in contrary with the result of the present study. The result of our study showed there was no statistical significance between Alfalfa and CPP-ACP, thus, Alfalfa can be considered as an economical herbal substitute to CPP-ACP.

Conclusion

Within the limitations of this invitro study, the following inference was drawn

- Each test group, when compared with the control group, showed a significant difference existing for element Ca and P
- Group A(alfalfa) and group B(CPP-ACP) showed a statistically significant increase in remineralization when compared to group C (F. Varnish)
- EDAX was found to be an efficient way to quantitatively assess the change in mineral content during in vitro caries studies.

Thus, the present study concludes that Alfalfa is effective in remineralizing the artificial enamel subsurface lesion and can be considered as an economical substitute to CPP-ACP tooth mousse. A further study has to be focused on standardization of alfalfa and it is also necessary to exploit its maximum potential in the field of dental sciences for novel and fruitful application.

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