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Antimicrobial property and Cytotoxicity of hesperidin incorporated dentin adhesive – an invitro study

M. Shamly[1], Iffat Nasim[2*]

[1]Post graduate student, Department of Conservative dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University

[2]Head of the Department, Department of Conservative dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University

***Corresponding author:** Iffat Nasim, Head of the Department, Department of Conservative dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Email: iffatnasim@saveetha.com

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ABSTRACT

Introduction: Dentin adhesives are frequently used in restorative dentistry, although they can be vulnerable to bacterial colonisation, which can result in recurrent caries. Citrus fruits contain the flavonoid hesperidin (HPN), which has demonstrated potential antibacterial activity against a variety of microorganisms. This study looked at the cytotoxicity and antibacterial effects of total etch dentin adhesive that was incorporated with hesperidin.

Materials and Methods: Four groups of dentin adhesive formulations with different hesperidin concentrations (25µL, 50µL, and 100µL) were made. The test microbes for the antimicrobial investigation were Lactobacillus acidophilus, Streptococcus mutans, and Enterococcus faecalis. For this experiment, Mueller Hinton Agar was used to measure the zone of inhibition. Also, the cytotoxicity of the hesperidin-incorporated total etch dentin adhesive was examined using the Brine Shrimp Lethality Assay.

Results: Dimethyl sulfoxide (DMSO), total etch bonding agent, and HPN work together synergistically to combat L. acidophilus, E. faecalis, and S. mutans in particular. Hesperidin concentration in the adhesive led to an expansion of the zone of inhibition. The hesperidin-incorporated adhesive did not have any harmful effects, as shown by the Brine shrimp lethality experiment.

Conclusion: According to the results of this in vitro investigation, adding hesperidin to dentin adhesive may improve its antibacterial capabilities without harming dental pulp stem cells. These findings open up new directions for investigation into the development of dental adhesive formulations with enhanced antibacterial characteristics for superior clinical results.

Clinical Significance: Hesperidin and other natural substances could be used into dentin adhesive to prevent bacterial colonisation and avoid recurrent caries. Furthermore, the hesperidin-incorporated adhesive's lack of cytotoxicity proves that it is safe for usage in clinical settings.

Keywords: *Hesperidin, Flavonoid, Total etch dentin adhesive, Micro-organisms, Quality of life*

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INTRODUCTION

Dental caries is a prevalent oral health issue that affects people of all ages worldwide. Dentin adhesive agents are commonly used in restorative dentistry to establish strong bonds between the tooth structure and restorative materials1. However, these adhesives are susceptible to bacterial colonization, leading to recurrent caries and treatment failures. The dentin-resin hybrid layer is regarded as the weak link in resin-based restorations2. Unpolymerised resins may affect the hybrid layer, may be sensitive to dentin and may alter the pulpal physiology. Flavonoids acts as a natural collagen cross linker in dentin.Incorporating natural compounds, such as flavonoids, into dentin adhesive formulations has emerged as a promising approach to improving their antimicrobial properties and reducing the risk of recurrent caries3. Total etch dentin adhesives are preferred because they are proved to have considerable bond strength compared to self etch dentin adhesives4. However, there are certain drawbacks, like collagen deterioration from acid etching5. MMPs and cysteine cathepsins , called pro enzymes that are produced from the host, are inactive6. They become active at lower pH levels as a result of acid conditioning, which can lead to the destruction of collagen, elastin, and extracellular matrix (ECM)7. This has an impact on the hybrid layer and reduces stability of the composite resin's bonds8. Furthermore, etching gelatinizes collagen fibres and limits resin diffusion in interfibrillar regions. Collagen fibres that aren't protected can then degrade. Its degradation can be avoided by adding substances with collagen crosslinking and MMP inhibitory properties to total etch9. A class of plant substances called flavonoids are well known for being anti-inflammatory and antioxidant. It has been demonstrated that they may be utilised to enhance the qualities of dental materials, particularly adhesives used in restorative dentistry.

Hesperidin, a flavonoid compound found in citrus fruits, has received considerable attention for its potential health-promoting properties10. Recent studies have highlighted the antimicrobial activity of flavonoids against various bacteria, including Streptococcus mutans and Lactobacillus acidophilus, which are the primary causative agents of dental caries1112. Hesperidin possesses anti-inflammatory and antioxidant properties, making it an ideal candidate for use in dental adhesives. The incorporation of hesperidin into dentin adhesive may show promising results in improving its antimicrobial properties13,14. Several studies have reported that the addition of Hesperidin has minimal cytotoxic effects on human cells, making it a safe and effective additive for dental adhesives.

The objective of this study was to investigate the antimicrobial property and cytotoxicity of hesperidin-incorporated total etch dentin adhesive using in vitro assays. Specifically, we aimed to evaluate the effect of varying concentrations of hesperidin (25µL, 50 µL ,100 µL) on bacterial growth and dental pulp stem cell viability.The findings of this study may provide valuable insights into the development of new dental adhesive formulations with enhanced antimicrobial properties for better clinical outcomes. The incorporation of natural compounds, such as hesperidin, into dentin adhesive could provide a safe and effective way to combat bacterial colonization and prevent recurrent caries. The results of this study may pave the way for further research to optimize the concentration and delivery of hesperidin in dental adhesive formulations to improve the quality of life by providing long lasting restorations. The null hypothesis states that there is no discernible difference between commercial dentin adhesive and dentin adhesive with hesperidin. Our team has extensive knowledge and research experience that has translate into high quality publications15–24,25–29

MATERIALS & METHODS

Preparation of test solution

2% of Hesperidin(HPN)is incorporated into dentin adhesive(2mg powder in 98ml of bonding agent). Adper single bond 2 was the total etch adhesive used in this study. 2 % HPN provides immediate bond strength and does not produce discoloration. Dimethyl sulphoxide was used in little amount as a solvent to solubilize hesperidin. Hesperidin powder (Sigma–Aldrich) was directly dissolved into pure Dimethyl sulfoxide (20mg of HPN in 0.025ml of

DMSO).The parent material was Adper Single Bond 2, a total etch dentin adhesive that is sold over the counter (3 M ESPE). Then the Hesperidin/Dimethyl sulfoxide was incorporated into Adper single bond 2 at proper ratio (20mg of HPN in 1 ml of bonding agent) to get the final concentration 2% hesperidin in the total etch adhesive employed.

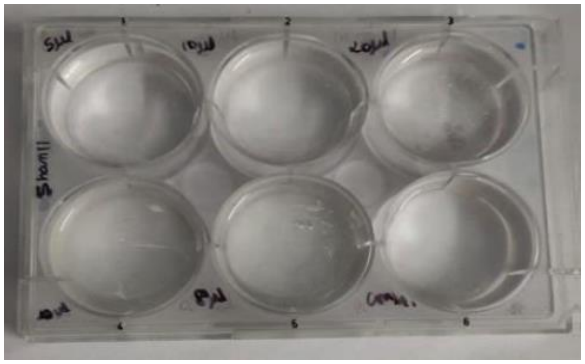
Control group: Flavonoid(HPN)free adhesive

Test group: Flavonoid(HPN) incorporated adhesive (20mg HPN+0.025ml DMSO+ 1ml of Adper single bond 2)

Antimicrobial activity

In order to identify the zone of inhibition, Mueller Hinton Agar was used.

o The test organisms were swabbed after the wells were cut with a 9 mm sterile polystyrene tip.



Brine Shrimp Lethality Assay

RESULTS

Measuring the diameter of the zone of inhibition surrounding the substance that was tested against the target microbiological organism is necessary for interpreting the zone of inhibition assay, a technique used to evaluate a substance's antimicrobial efficacy. The zone of inhibition is the region around the substance where the microbe's growth has been impeded or ceased. The effectiveness of the chemical against the microorganism increases with the diameter of the zone of inhibition. The type of organism being tested, the chemical being utilised, and the substance's concentration can all affect the

o Several concentrations of the experimental adhesive (25, 50, and 100 mL) were loaded, and standard antibiotic amoxyrite was injected in the fourth well.

The plates were incubated at 37 °C for 24 hours. The zone of inhibition was assessed following the incubation period.

Assessment of cytotoxicity

o Ten to twelve millilitres of saline water were added to six-well ELISA plates. A total of 10 nauplii (5, 10, 20, 40, 80, and control-normal saline) were gradually added to each well.

Thereafter, in accordance with the concentration level, the experimental adhesive solutions were added. 24 hours were spent incubating the plates.

o The ELISA plates were examined after 24 hours and counted for the presence of live nauplii.

breakpoints for interpreting the zone of inhibition assay.

With increasing hesperidin-incorporated dentin adhesive concentration, the zone of inhibition expands. The most frequent cariogenic microbes, lactobacillus acidophillus and streptococcus mutans, show the greatest inhibition. Enteroccus faecalis exhibits significantly reduced inhibition. The following figures (a,b,c) and table(1) show the outcomes.

Zone of inhibition in relation to Lactobacillus acidophillus

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Zone of inhibition in relation to Enterococcus faecalis

Zone of inhibition in relation to Streptococcus mutans

TABLE 1: Measurement of Zone of inhibition of test and control groups

	Control	Test (25µL)	Test (50µL)	Test (100µL)
L.acidophilus	13mm	14mm	16mm	18.5mm
E.faecalis	9mm	10mm	12mm	14mm
S.mutans	10mm	11mm	12mm	13.5mm

The bioassay known as the brine shrimp lethality assay is frequently used to assess the toxicity of chemical substances. The dead shrimp in each concentration of the substance under test is counted in order to evaluate the assay's results. The relationship between a chemical's concentration and a response is known as a dose-response relationship (in this case, shrimp mortality). The toxicity of the drug and the success of the assay can both be determined using this connection. The concentration of a substance at which 50% of shrimp die is known as the LC50 (lethal concentration 50). The higher LC50 in this investigation indicates reduced cytotoxicity. Table 2 presents the findings.

TABLE 2: Assessment of live nauplii's in relation to concentration of hesperidin incorporated into dentin adhesive

Concentration (µL)	No. of live nauplii's
5	9
10	8
20	7
40	7
80	7
Control	10

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DISCUSSION

Flavonoids have antimicrobial properties, collagen-crosslinking effects, and dentin collagen proteolytic degradation inhibition30. The mechanical properties of dentine are improved by the cross-linking of collagen and other proline-rich proteins by a naturally occurring flavonoid called Hesperidin. Yet, depending on the chemical composition of the flavonoids and the type of microbe, different flavonoids have different antibacterial potencies31. When exposed to flavonoids like hesperidin, several bacteria become immune to infection and multiplication32. Molecular structure, hydrophobicity, solubility, the presence or absence of a sugar moiety, and the kind of sugar in the chemical backbone all have an impact on the antibacterial activity and bioavailability of flavonoids33.Yet, it is still unknown exactly how flavonoids work to prevent bacteria from growing. The interruption of bacterial DNA synthesis, bacterial motility, cytoplasmic membrane permeability, and inhibition of bacterial metalloenzymes are only a few of the methods that have been put forth. Two flavonoids from the Citrus genus, hesperidin (Hsd) and hesperetin (Hst), exhibit a range of biological activities, including antioxidant, anti-inflammatory, and anti-cancer effects34. Also, it's possible that some strains of Gram+ and Gram-bacteria, including Staphylococcus aureus, are immune to the antimicrobial effects of glycoside flavones34,35. The many effects of flavonoids, such as hesperidin, on healthy dentin have opened up a new avenue for the development of therapeutic medicines to support bonding stability on caries-affected dentin.

When Islam et al. (2012) looked at how adding natural cross-linkers affected the resin-dentine bond strength of a self-etching adhesive, they discovered that adding HPN significantly increased micro-TBS, adding grape seed extract (GSE) significantly decreased micro-TBS, and adding chlorhexidine (CHX) had no statistically significant differences36. Their findings demonstrate that the micro-TBS and mechanical characteristics of the bonded interface were immediately enhanced by the addition of HPN to the Clearfil SE primer. Ghorab et al. (2018) examined the antimicrobial activity and adhesive properties of a condensed total-etch adhesive system containing varying amounts of Hesperidin (HPN), coming to the conclusion that doing so significantly increased the dental adhesive's immediate TBS at 0.2 wt percent and 0.5 wt percent HPN (P 0.05)32. Thermocycling significantly reduced the TBS of dental adhesives with 0.5 wt percent HPN incorporated, which showed a potential antibacterial impact without changing the adhesive properties.

When combined with DMSO and added to a total etch bonding agent, hesperidin is very efficient against L. acidophilus, S. mutans, and E. faecalis37. The adhesive characteristics of total etch dentin adhesive are not adversely affected when HPN is applied at 2% concentration, but a promising antibacterial effect is attained. Hesperidin contains collagen cross-linking and MMP inhibitory activities. As a result, dentin adhesive's initial bond strength may be improved3839. Hesperidin can be more easily dissolved with the use of dimethyl sulfoxide (DMSO). DMSO enhances adhesive penetration into the exposed collagen matrix after acid conditioning, which enhances adherence40. In addition, DMSO has the power to inhibit the collagen-degrading enzymes that are obtained from the host. The hybrid layer's durability is enhanced as a result. Biocompatible adhesive systems are those that establish a solid bond with biological tissues and permit both tissue repair and tissue differentiation. In the current investigation, the cytotoxicity of the experimental adhesive was affected by its concentration. But those standards are still respectable. Hesperidin (HPN) functions as a natural collagen cross-linker that aids in guarding against exposed collagen deterioration brought on by acid etching. The degree of conversion of unreacted monomers is improved by its antioxidant property41. This naturally occurring flavonoid called Hesperidin has a chroman ring that can interact with proteins high in proline, such collagen, to cross-link them and enhance the mechanical qualities of dentin. As a result, it aids in enhancing the biocompatibility and sealing capacity of dentin adhesives.

CONCLUSION

Within the constraints, it can be said that adding 2% hesperidin to total etch dentin adhesive could achieve therapeutic objectives that are important for biocompatibility without impairing dentin adhesive's adhesive capabilities. Moreover, it is antibacterial. Hence, it can be utilised to prevent the development of secondary caries in adhesive restorations.

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