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DIEGO MARTINS DE PAULA

APLICAÇÃO DA BIOMASSA RESIDUAL DA INDÚSTRIA DO PAPEL NA ODONTOLOGIA ADESIVA

FORTALEZA

2019

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, como requisito parcial à obtenção do título de doutor em Odontologia. Área de concentração: Clínica Odontológica.

Orientador: Prof. Dr. Victor Pinheiro Feitosa.
Coorientador: Prof. Dr. Diego Lomonaco

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A Deus.

Aos meus pais, Antônio Justino de Paula e Maria Amélia Martins de Paula.

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RESUMO

Lignina é um polímero natural proveniente do resíduo de descarte da indústria do papel, com grande potencial para formação de ligações cruzadas com as fibras de colágeno da dentina pela sua estrutura química. Deste modo, esta tese, constituída por 2 artigos, teve por objetivos: (1) avaliar a capacidade biomodificadora da Lignina na dentina humana desmineralizada testada como pré-tratamento de restaurações de resina composta; (2) avaliar os efeitos da incorporação de agentes biomodificadores naturais incorporados no ácido fosfórico na resistência de união à microtração (μTBS) e nanoinfiltração (NL) de um adesivo convencional de dois passos. No estudo 1, soluções experimentais com Proantocianidina 6,5% (PAC), Cardanol 2% (CARD), Lignina (LIG) nas concentrações de 1, 2, 4% (LIG1, LIG2, LIG4) foram manipuladas. O controle negativo foi a solução hidroetanólica sem agente natural (CN). Os testes quantitativos utilizados após as restaurações de resina composta utilizando as soluções como pré-tratamento (1 minuto) do sistema adesivo foram a μTBS (n=7) testados 24h e após termociclagem, grau de conversão (GC) *in situ* (n=3) da interface de união e qualitativos a NL e micropermeabilidade em microscopia Confocal. Barras de dentina desmineralizada foram utilizadas para os testes quantitativos de flexão de três pontos (n=12) antes (baseline) e após da biomodificação de 1 minuto, variação de massa (n=12) após 48h e 4 semanas do tratamento, teste de Hidroxiprolina (HYP) após 4 semanas de degradação em água, e para análise qualitativa pela espectroscopia micro-Raman (n=3) dos picos formados após o tratamento biomodificador. No estudo 2, ácido fosfórico 37% experimental foi manipulado e incorporado com 2% de agentes biomodificadores: LIG, CARD e PAC. O CN foi o ácido sem agente biomodificador e o ácido controle comercial (CC) também foram utilizados. A espectroscopia de infravermelho transformada Fourier (FTIR) foi usada para avaliar a formação de ligações cruzadas em superfícies de dentina condicionadas por 15s com cada ácido. O teste quantitativo μTBS (n=7) com análise do padrão de fratura (24 horas e envelhecida) e a análise qualitativa da NL também foram realizados. Os resultados de ambos os estudos foram analisados com ANOVA ou ANOVA de medidas repetidas e pós-teste de Tukey ($p<0,05$). Os resultados do estudo 1, LIG e PAC mantiveram suas μTBS, as imagens de NL e micropermeabilidade mostraram proteção da camada híbrida. Somente o LIG1 não reduziu o GC *in situ*. LIG2, LIG4 e CARD aumentaram o módulo de elasticidade das barras de dentina desmineralizadas. LIG1 e LIG2 reduziram a perda de massa da barra de dentina. Os picos 1117 cm^{-1} e 1235 cm^{-1} (biomodificação) foram mostradas com micro-Raman. LIG1 liberou menos HYP. Os resultados do estudo 2 mostraram picos de ligação cruzada no FTIR.

LIG e CARD mantiveram a μTBS e mostraram uma redução de prata na NL após o envelhecimento ocorreram mais fraturas adesivas. Portanto, LIG foi capaz de realizar ligações cruzadas com as fibras colágenas desmineralizadas aplicada como pré-tratamento e incorporadas no ácido fosfórico, sugerindo que possa prevenir a degradação da dentina condicionada e desprotegida.

Palavras-chave: Lignina. Dentina. Colágeno. Restauração Dentária Permanente. Odontologia

ABSTRACT

Lignin is a natural polymer not tested in restorative dentistry with great potential for crosslinking with dentin collagen fibers. The aim of this thesis was: (1) to evaluate the cross-linker capacity of Lignin in the demineralized human dentin assayed as pre-treatment of composite resin restorations; (2) to evaluate the effects of the incorporation of natural cross-linkers in phosphoric acid into the microtensile bond strength (μTBS) and nanoleakage (NL) to two-step etch&rinse adhesive. In study 1, experimental solutions with Proanthocyanidin 6.5% (PAC), Cardanol 2% (CARD), Lignin (LIG) at concentrations of 1, 2, 4% were manipulated. The negative control was the solution without cross-linkers (NC). Quantitative tests, used after the composite resin restorations using the pretreatment solutions (1-minute), were μTBS (n=5) tested 24h and after thermocycling, *in-situ* conversion degree (DC, n=3) of hybrid layer and qualitative tests to NL and micropermeability in Confocal microscopy. Dentin bars were used for quantitative tests three points bending test (n = 12) before (baseline) and after 1-minute biomodification; mass variation (n=12) after 48 hours and 4 weeks of treatment; Hydroxyproline (HYP) test after 4 weeks of degradation in water and for qualitative analysis by micro-Raman spectroscopy (n=3) of the peaks formed after the biomodification treatment. In study 2, experimental phosphoric acids were manipulated and incorporated with 2% of cross-linkers: LIG, CARD and PAC. NC without cross-linkers and commercial control (CC) were also used. FTIR spectroscopy was used to detect collagen cross-linking in etched dentin specimens. The quantitative test μTBS (n = 7) with analysis of the fracture mode (24 hours and aging) and the qualitative analysis of NL were also performed. The results of both studies were analyzed with ANOVA or ANOVA of repeated measurements and Tukey's post-hoc ($p<0.05$). The results of study 1, LIG and PAC maintained their μTBS, NL and micropermeability images showed hybrid layer protection. Only LIG1 did not disturb the GC in situ. LIG2, LIG4 and CARD increased the modulus of elasticity of the demineralized dentin bars. LIG1 and LIG2 reduced dentine bar mass loss. Peaks 1117 cm^{-1} and 1235 cm^{-1} (cross-linking) were shown with micro-Raman. LIG1 released less HYP. The results of study 2 showed peaks of cross-linking in FTIR analysis. LIG and CARD maintained μTBS and showed little silver in NL after aging. The adhesive fracture mode was higher. Therefore, LIG was able to perform cross-links with the demineralized collagen fibers applied as pre-treatment and incorporated into phosphoric acid, preventing the degradation of unprotected dentin and increasing the longevity of the restorations.

Keywords: Dentin. Lignin. Cardanol. Proanthocyanidin. Cross-Linking Reagents

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1 INTRODUÇÃO GERAL

O substrato dentinário é composto em volume por 20% de água, 50% de tecido mineralizado e 30% de matriz orgânica extracelular. Esta última é representada em 90% pelo colágeno tipo I e 10% de componentes não-colagenosos como proteoglicanas, glicosaminoglicanas e proteases endógenas, dentre elas as metaloproteinases de matriz (MMPs) e catepsinas cisteínicas (GOLDBERG et al., 2011; AGUIAR, et al., 2014; BEDRAN-RUSSO; RAVINDRAN; GEORGE, 2013). As proteoglicanas têm um importante papel na mineralização do tecido dentinário, além de serem as responsáveis pela integridade estrutural das fibras colágenas (BEDRAN-RUSSO et al., 2014; VIDAL et al., 2017). Já as proteases endógenas são enzimas que degradam as fibras colágenas desmineralizadas (PASHLEY et al., 2011). A dentina é o substrato dental mais susceptível pela cárie por causa da sua limitada capacidade de regeneração (BEDRAN-RUSSO; RAVINDRAN; GEORGE, 2013), assim deve-se empregar estratégias para reduzir a sua degradação.

A degradação endógena das fibras colágenas acontece porque a dentina desmineralizada expõe enzimas hidrolíticas como as MMPs (2, 3, 8, 9 e 20) e provoca a liberação de catepsinas que, uma vez ativadas podem lentamente degradar as fibrilas colágenas não infiltradas pelos monômeros na camada híbrida (PASHLEY et al., 2011). Outra forma de degradação é hidrolítica, onde a presença de água da própria dentina, a resultante da lavagem após condicionamento ácido e até mesmo a que está presente na composição do sistema adesivo são responsáveis pela degradação das fibras colágenas desmineralizadas (BRESCHI et al., 2008).

A biomodificação do substrato dentinário é de uma abordagem da Odontologia Restauradora que dá ênfase ao aumento da densidade de ligação cruzada do colágeno para melhorar as propriedades biomecânicas da dentina e reduzir a sua taxa de biodegradação. Isso acontece porque esse aumento das ligações reduz a penetração de água na camada híbrida,

além de resultar numa redução da mobilidade molecular das MMPs, também podendo evitar ligações químicas das proteases em seus sítios receptores (BEDRAN-RUSSO et al., 2008, BEDRAN-RUSSO et al., 2009; AL-AMMAR A; DRUMMOND; BEDRAN-RUSSO, 2009; PASHLEY et al., 2011).

Estratégias de biomodificação dentinária podem ser a partir de produtos químicos sintéticos ou naturais (BEDRAN-RUSSO et al., 2014). Alguns agentes químicos sintéticos como o glutaraldeído já são utilizados na odontologia há décadas. O glutaraldeído é um dialdeído com alta afinidade com radical da amina primária dos aminoácidos. A sua desvantagem é ser citotóxico após sua lixiviação do tecido tratado (AL-AMMAR A; DRUMMOND; BEDRAN-RUSSO, 2009; PASHLEY et al., 2011; BEDRAN-RUSSO et al., 2011; BEDRAN-RUSSO et al., 2014). A carbodiimida é outro aldeído que funciona como agente biomodificador menos tóxico que o anterior. O seu mecanismo acontece por causa da ativação dos grupos ácidos carboxílicos dos ácidos glutâmico e aspártico que se ligam ao grupamento amina da lisina para ligação cruzada amida. A sua desvantagem é seu limitado potencial para realizar ligações cruzadas (BEDRAN-RUSSO et al., 2010; BEDRAN-RUSSO et al., 2014; VIDAL et al., 2016). Outros agentes químicos sintéticos agem por ligação em receptores específicos, como a galardina (BRESCHI et al., 2010), ou não-específicos, como a clorexidina (LOGUERCIO et al., 2009), podem ser utilizados previamente às restaurações para prevenir a degradação da camada híbrida, porém possuem sua ação limitada com o passar do tempo (LOGUERCIO et al., 2009; VIDAL et al., 2016).

A utilização de agentes naturais para a biomodificação da dentina é uma alternativa atrativa, econômica e renovável de interesse para a utilização em procedimentos clínicos (AL-AMMAR A; DRUMMOND; BEDRAN-RUSSO, 2009). Extratos de plantas ricos em polifenóis têm mostrado uma efetiva bioatividade com componentes da matriz celular e extracelular e são produtos promissores para aplicações na odontologia (VIDAL et al., 2014).

Alguns exemplos são extratos das sementes da uva (*Vitis vinifera*) e do cacau (*Theobroma cacao*), folhas da planta chá verde (*Camellia sinensis*), casca do caule da canela (*Cinnamon verum*), da fruta açaí (*Euterpe precatoria*) e aroeira (*Myracrodruon urundeuva*) (AGUIAR *et al*., 2014). Cardanol e cardol são fenóis de cadeia de carbono longa obtidos da extração industrial do líquido da castanha de caju (*Anacardium occidentale*) e já foram documentados na literatura como agentes biomodificadores naturais do colágeno da dentina (MOREIRA et al., 2017; MOREIRA et al., 2018).

A lignina é um polímero natural de origem vegetal ainda não investigado na Odontologia. Pela sua estrutura química, a lignina deve possuir um grande potencial para formação de ligações cruzadas com as fibras de colágeno da dentina, o que favorece uma proteção contra a sua biodegradação. Assim, possibilitaria uma melhora na longevidade das restaurações de resina. A ideia de se utilizar esse polímero natural para este fim surgiu a partir do estudo da diferenciação da estrutura da parede celular vegetal, que fornece suporte mecânico aos seus tecidos. Juntamente com a celulose e hemicelulose, a lignina é o componente principal das paredes celulares secundárias. A deposição da lignina inicia-se após o término da diferenciação celular. Ela preenche os poros entre os polissacarídeos nas paredes celulares, levando a um espessamento secundário das paredes e aumento da sua rigidez, fato este importante para proteger as plantas contra os ataques de agentes patogênicos e do estresse mecânico (SAKAGAMI, 2014; YOON; CHOI; AN, 2015).

O aumento na resistência das paredes celulares vegetais se dá através de ligações químicas da lignina com a celulose e hemicelulose (YOON; CHOI; AN, 2015). Isso é passível de acontecer com fibras colágenas da dentina por causa da estrutura química da lignina que consiste de subunidades monoméricas como o p-hidroxifenil (H), guaiacil (G) e siringil (S). Esses polifenóis teoricamente são passíveis de realizar pontes de hidrogênio ou outras ligações covalentes com os peptídeos do colágeno (SAKAGAMI, 2014). Hoje em dia, a

lignina é considerada um subproduto do processo de fabricação de celulose (obtido a partir do licor negro resultado no processo de cozimento da madeira), sendo utilizado pela indústria para a geração de energia. Também pode substituir derivados de petróleo em aplicações de alto valor agregado, em indústrias e segmentos diversos (MATHEWS; SMITHSON; GRUNDEN, 2016). Entretanto, a sua aplicação odontológica pode gerar bons resultados e gerar uma finalidade sustentável para esse produto de descarte industrial.

2 PROPOSIÇÃO

O presente trabalho tem como objetivos:

2.1 OBJETIVO GERAL

Avaliar a lignina proveniente da indústria do papel como agente biomodificador na dentina humana desmineralizada para a odontologia adesiva.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a capacidade da lignina de realizar ligações cruzadas com a dentina desmineralizada aplicada como pré-tratamento no procedimento adesivo das restaurações de resina composta;

- Avaliar a capacidade da lignina de realizar ligações cruzadas com a dentina desmineralizada aplicada incorporada no ácido fosfórico no procedimento adesivo para restaurações de resina composta;

- Avaliar a capacidade da lignina proteger a camada híbrida das restaurações de resina composta após o envelhecimento;

- Avaliar a capacidade da lignina aumentar as propriedades mecânicas do colágeno desmineralizado;

- Avaliar a capacidade da lignina proteger o colágeno contra degradação.

- Comparar a capacidade biomodificador da dentina com outros agentes biomodificadores.

3 CAPÍTULOS

Esta tese está baseada no Artigo 46 do Regimento Interno do Programa de Pós-graduação em Odontologia da Universidade Federal do Ceará que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato (Anexo 1). Por se tratarem de pesquisas envolvendo dentes humanos, os projetos de pesquisas destes trabalhos foram submetidos à apreciação do Comitê de Ética em Pesquisa da Universidade Federal do Ceará, tendo sido aprovados (Anexos 2). Assim sendo, esta tese é composta de dois capítulos contendo artigos publicados, submetidos para publicação em revistas científicas ou em fase de redação, conforme descrito abaixo:

Capítulo 1

"Collagen cross-linking of lignin improves etch-and-rinse dentin bonds"

De-Paula DM, Lomonaco D, Ponte AMP, Lima MP, Cordeiro KE, Feitosa, VP.

A ser submetido ao periódico Journal of Dental Research.

Capítulo 2

"Influence of lignin incorporation in phosphoric acid on dentin biomodification and bonding of an etch-and-rinse adhesive" De-Paula DM, Lomonaco D, Ponte AMP, Cordeiro KE, Feitosa, VP.

A ser submetido ao periódico Dental Materials.

3.1 CAPÍTULO 1

Collagen cross-linking of lignin improves etch-and-rinse dentin bonds

ABSTRACT

Cross-linking dentin collagen enhances mechanical stability and diminishes degradation, but no natural agent provided optimal outcomes so far. Goal was to evaluate cross-linking capacity of lignin in demineralized dentin, and its effects for adhesive composite restorations. Experimental hydroethanolic solutions with cross-linkers proanthocyanidins 6.5% (PAC, from grape-seed extract), Cardanol 2% (CARD, from cashew-nut shell liquid) and lignin (LIG, from Eucalyptus) at 1, 2 or 4% concentrations were prepared. Negative control (NC) was only the hydroethanolic solution. Extracted third molars were cut and randomized for the dentin microtensile bond strength (μTBS) test assayed after 24h or 1000 thermal cycles. Further resin-dentin specimens were used for nanoleakage assessment in SEM, micropermeability survey in Confocal microscopy and in situ degree of conversion (DC) analysis by Micro-Raman spectroscopy. Other molars were sectioned to obtain demineralized dentin bars tested in three-point bending to obtain elastic modulus (E) before and after 1-minute biomodification. Mass change of treated-bars was tracked until 4-weeks water storage, whilst hydroxyproline (HYP) release was investigated in storage solution by UV-Vis spectroscopy. Vibrational crosslinking was surveyed by Micro-Raman spectroscopy. The results were analyzed by ANOVA and Tukey's test (α=0.05). Reduction on μTBS was observed in NC ($p<0.001$) and CARD ($p=0.026$). LIG4 increased μTBS after aging ($p=0.022$). Nanoleakage micrographs showed hybrid layer protection with all agents, but reduced micropermeability was attained only with LIG2 and LIG4. Polymerization was reduced with all cross-linkers, except LIG1. LIG2, LIG4 and CARD increased E, whereas LIG1 and LIG2 reduced the mass loss after degradation. Raman spectra depicted effective collagen crosslinking (peaks 1117cm-1 and 1235cm-1) for all agents. HYP release was significantly lower for LIG1 than NC ($p <0.001$). Lignin is sustainable plant-derived product able to optimally perform collagen cross-linking and improve adhesion stability of composite resin restorations, thereby preventing the degradation of unprotected dentin collagen.

Keywords: Dentin. Lignin. Cardanol. Proanthocyanidin. Cross-Linking Reagents

INTRODUCTION

In order to attain durable bonding for direct and indirect restorations, the formation of uniform and well-sealed hybrid layer is essential (Nakabayashi et al. 1982). Such structure, in dentin, relies on an entangled mesh of polymers and collagen fibrils that promotes micromechanical interlocking (Nakabayashi and Saimi 1996). Nevertheless, thanks to the less mineralized and hydrated nature of dentin (Goldberg et al. 2011), this substrate is harsh to be infiltrated with hydrophobic monomers, leaving several collagen fibrils unprotected, thereby triggering bonding failures and diminishing their longevity (Breschi et al. 2008; Al-Ammar et al. 2009).

In modern Restorative Dentistry, many strategies were studied to accomplish dentin biomodification with collagen cross-linking (Gu et al. 2018) by means of enzymatic and non-enzymatic chemical reactions, augmenting tensile strength and elastic modulus of demineralized dentin (Al-Ammar et al. 2009; Vidal et al. 2016). This could increase the durability of composite restorations. Such cross-links reduce endogenous proteolysis undertaken by matrix metaloproteinases (MMPs), and protect hybrid layer from hydrolysis (Breschi et al. 2008; Bedran- Bedran-Russo et al. 2010, Bedran-Russo et al. 2011; Bedran-Russo et al. 2014; Castellan et al. 2010). The biomodification strategy relies on the application of synthetic or natural compounds in dentin before or during restorative procedure (Bedran-Russo et al. 2014). Synthetic agents, such as glutaraldehyde and carbodiimide, afford highly reticulated cross-links, but the clinical application remains questionable due to potential cytotoxic reactions (Bedran-Russo et al. 2014; Hass et al. 2016).

Natural cross-linkers are attractive because they originate from renewable and sustainable sources, with further interests in global outlook (Aguiar et al. 2014; Moreira et al. 2017). Most of these agents are polyphenols, with proanthocyanidins (PACs) from grape-seed

extract (*Vitis vinifera*) been highlighted for noteworthy outcomes demonstrated in several investigations (Castellan et al. 2010; Vidal et al. 2014; 2016, Leme-Kraus et al. 2017). Particular mechanisms of cross-linking are speculative, with reports about hydrogen bonds, covalent links and hydrophobic interactions (Liu et al. 2014; Aguiar et al. 2014; Vidal et al. 2016). More recently, long-carbon chain cardanol and cardol, from industrial residue of cashew nut (*Anacardium occidentale)* production, demonstrated optimal biomodification capacity (Moreira et al. 2017; Moreira et al. 2018).

Following sustainability line, lignin (*Eucalyptus*), a polyphenol-rich natural polymer, is the main component of secondary plant cell walls, which supplies rigidity to vegetal architecture by cross-linking cellulose and hemi-cellulose (Sakagami 2014; Yoon et al. 2015). Its obtainment occurs from black liquor produced during wood cooking in industrial production of paper. Although its promising chemical structure and high production worldwide, to our knowledge, lignin was never investigated in adhesive dentistry (Sakagami and Tomomura 2018).

Therefore, the aim of this study was to survey biomodification ability of lignin in human dentin and its effects as pre-treatment for an etch-and-rinse adhesive. Study hypothesis is that lignin attains similar cross-linking to PAC and cardanol, due to potential chemical structure (Fig.1)

MATERIALS AND METHODS

Preparation of biomodification solutions

Cardanol (CARD) was obtained from cashew-nut shell liquid, donated by Amêndoas do Brasil LTDA (Fortaleza, Brazil). Cardanol and Lignin were separated and purified by the methods described in supplement (Lomonaco et al, 2013). Cardanol was diluted in

water/ethanol (1:1) at 2wt% concentration. PAC solution was prepared by dissolving 6.5wt% grape-seed extract (Meganatural Gold, Madera, USA) in water/ethanol (1:1) with 5min stirring at 25ºC and double-filtering. Lignin (LIG) was supplied by paper industry Suzano SA (Limeira, Brazil). It was diluted in water/ethanol (1:1) at 1, 2 or 4wt% concentrations. All solutions were buffered to pH 7.2. Hydroethanolic solution was employed to standardize the dissolution of cardanol and lignin, which possess low solubility in only distilled water. As negative control (NC), the water/ethanol (1:1) solution without biomodification agent was used. CARD and LIG were characterized according to Moreira et al. 2017 by NMR. Chemical structures of agents are shown in Figure 1.

Bonding Procedures

Extracted human third molars were used after approval of institutional Ethics Committee (protocol 011133/2018). Medium dentin surfaces were obtained by sectioning occlusal enamel with diamond saw in a cutting machine (Cutmaster, Londrina, Brazil). Exposed dentin surfaces were abraded with wet 320-grit SiC papers for 30s to standardize the smear-layer.

The dentin was etched with 37% phosphoric-acid gel (Condac37, FGM, Joinville, Brazil) for 15s, rinsed with distilled water for 30s, biomodification solutions were applied for 60s and rinsed for 20s with distilled water. Dentin was left moist and the two-step etch-and-rinse adhesive Optibond S (Kerr, Orange, USA) was actively applied in two coats for 20s, gently air-dried for 3s and light-cured for 20s with LED unit Valo (1200mW/cm², Ultradent, South Jordan, USA). Two 2mm-thick layers of resin composite Opallis (FGM) were placed and individually light-cured for 40s. Bonded teeth were stored in distilled water for 24h at 37ºC.

Microtensile Bond Strength (μTBS) and Failure Pattern

Bonded teeth (n=7) were longitudinally sectioned in resin-dentin sticks with approximately 1mm² cross-sectional area. Half of sticks per tooth were tested immediately and the other half was tested after 1,000 thermal cyclings (30s in 5ºC and 30s in 55ºC with 10s interval) (Huber-Mechatronik TC45SD, SD Mechatronik, Feldkirchen-Westerham, Germany) (Perote et al. 2015). μTBS test was performed by attaching the resin-dentin sticks in Geraldeli's jigs with cyanoacrylate cement, adapted in a Microtensile Machine (OM-100, Odeme, Luzerna, Brazil) and tested until failure with 0.5mm/min crosshead speed. Prior to the test, each stick had cross-sectional area measured with digital calliper to obtain the bond strength in megapascals (MPa) (Castellan et al. 2013). Pre-test fractures were not often and were included as 0 MPa.

All fractured sticks were examined by steromicroscopy (60x magnification, Stereo-zoom S8, Leica, Heidelberg, Germany) to identify failure patterns, classified as adhesive, cohesive in dentin, cohesive in composite or mixed.

Nanoleakage

Two resin-dentin sticks from each subgroup were assessed for silver nanoleakage according to the protocol of Tay et al. (2002), using 50% ammoniacal silver nitrate solution. Briefly, specimens were immersed in tracer silver solution for 24h in darkness, rinsed with distilled water and immersed in photodeveloping solution for 8h under fluorescent light. They were then embedded in epoxy resin and polished with SiC papers up to 4000-grit and 1-μm diamond paste (Buehler, Coventry, UK) in polishing clothes. Stubs were cleaned for 5min in ultrasonic bath after each polishing step, and dehydrated for 24h in silica gel incubator at

37ºC. They were gold-sputter coated and observed in field-emission-gun scanning electron microscopy (Quanta FEG, FEI, Amsterdam, Netherlands) in backscattered electron mode.

Micropermeability Assay

Eighteen third molars (n=3) were restored as aforementioned, but using adhesive doped with 0.1wt% Rhodamine B (Sigma-Aldrich, St. Louis, USA). Bonded-teeth were perfused with 0.3wt% aqueous fluorescein (Sigma-Aldrich) solution under 15cm H_2O simulated pulpal pressure for 3h. Thereafter, they were cut in 1mm-thick slices, polished with 2000-grit wet SiC papers and ultrasonicated for 2min. Specimens were observed in confocal-laser scanning microscopy (LSM 710, Leica) following the setup of Feitosa et al. (2014).

In situ Degree of Conversion (DC)

To survey the influence of each biomodification agent on adhesive polymerization, the protocol of Navarra et al. (2016) was followed. In summary, restorative procedures were carried out as previously described, and bonded-teeth were then cut in 1mm-thick resin-dentin slabs. The specimens were positioned in Micro-Raman spectrophotometer (Xplora, Horiba, Paris, France) with 100x magnification lens (Olympus, London, UK) to obtain 1μm-beam diameter which was positioned in hybrid layer. Ratio of vibrational intensities of aliphatic C=C from methacrylate (1639 cm^{-1}) and the internal standard aromatic C=C (1609 cm^{-1}) were obtained from uncured adhesive and cured adhesive within hybrid layers. DC was calculated following the formula: DC=(1–R-cured/R-uncured) x 100, where R is ratio of peak heights of 1639cm^{-1} and 1609cm^{-1} vibrations. The analysis was realized in three slabs of each bonded-tooth and the results were averaged to obtain one statistical unit. Three bonded-teeth per group were tested (n=3).

Elastic Modulus (E)

Further forty-eight extracted molars were cut to obtain middle dentin bars with approximately 1mm-thick, 1mm-width and 7mm-length. The bars were demineralized in 10% phosphoric acid for 5h. They were randomly distributed within the five biomodification solutions and negative control (n=12). Three-point bending test was performed with 5N load cell, 0.5mm/min crosshead speed in a universal testing machine (Instron 3345; Instron Inc., Canton, USA) in untreated specimens (baseline) and after 60s immersion in each solution. Elastic modulus was obtained by the software (Bluehill, Instron) after 1mm displacement (Castellan et al. 2010).

Mass Change (Wmc)

The same demineralized dentin bars of elastic modulus experiment (n=12) were weighted in analytical balance (0.01mg precision, AUX-220, Shimadzu, Tokyo, Japan) before (M_1) and after (M_2) the immersion in solutions. Prior to weighing, they were individually dehydrated for 24h in vacuum desiccator with silica gel at 25ºC. Mass change (W_{mc}%) was determined by the percentage of gain/loss of mass of each specimen (Aguiar et al. 2014);

Biodegradation Rate (R)

The treated dentin bars (n=12) were individually stored in 1.5mL deionized water for 4 weeks to promote biodegradation after elastic modulus and mass change surveys. After such period, specimens were dehydrated as aforementioned and weighted (M_3). The protocol (Aguiar et al. 2014, Moreira et al. 2017) used to assess percentage biodegradation rate.

Hydroxyproline Assay (HYP)

The storage solutions of treated dentin bars, after the 4-week storage period, were joined to obtain three solutions with 6mL each. Aliquots of supernant were collected for testing with HYP assay kit (Sigma-Aldrich) following manufacturer's instructions. The final HYP-traced solutions were evaluated by UV-Vis spectroscopy (Ultrospec 1100 Pro, Amershan Biosciences, Little Chalfont, UK) with 550nm wavelength (Liu et al. 2014) to obtain absorbance, which was transformed to HYP concentration by means of standard curve solutions supplied in the kit.

Micro-Raman Cross-linking Identification

Further demineralized dentin bars (n=3) were investigated before and after 1-minute immersion in experimental solutions in Micro-Raman spectrophotometer (Xplora, Horiba) with 3.2 mW laser power and 632nm wavelength using 10s acquisition time and 3 accumulations. The spectra range was 700-1800 cm^{-1} to survey peaks and shoulders at 1117 cm^{-1} and 1235 cm^{-1} assigned to dentin collagen cross-linking (Moreira et al. 2017).

Statistical Analysis

Shapiro-Wilk normality test was used to assess normal distribution. After passing this test ($p>0.05$), data were statistically analyzed by ANOVA or repeated-measures ANOVA, and Tukey's post-hoc test ($\alpha=5\%$).

RESULTS

Outcomes of μTBS are depicted in Table 1. NC e LIG1 achieved the highest initial results without significant difference between them (p=0.059). All further groups showed statistical differences from each other. After thermocycling, NC (p<0.001) and CARD (p=0.026) dropped significantly the bond strength, whilst LIG4 increased (p=0.022). Further agents maintained μTBS stable (p>0.05). Most fractures were adhesive for all groups.

Nanoleakage micrographs are shown in Figure 2. Negative control depicted more intense silver uptake both in both 24h and after aging. Lignin in all concentrations provided similar nanoleakage exclusive in hybrid layer, with very few silver deposits after aging. Cardanol and PAC attained intermediary nanoleakage. Figure 3 depict representative images of micropermeability. Highest micropermeability was noted in NC and PAC-treated specimens whereas less permeable interface was attained with 4% lignin.

The presence of most biomodification agents decreased DC (Table 1), once all experimental groups, except LIG1, attained significantly lower conversion than negative control (p<0.05). No statistical difference (p=0.695) was found between NC and LIG1. Regarding elastic modulus (E), LIG4 yielded highest percentage increases on flexural modulus in comparison with further groups (Table 1). Conversely, absence of biomodification agents in NC promoted E reduction.

Lignin in lower concentrations (LIG1 and LIG2) achieved the best results (Table 1) in mass gain during 4-week water storage, with significant difference from negative control and PAC. HYP quantifications are shown in Table 1. Statistical lower HYP release was attained with LIG1 (0.33±0.01 μg/mL) in comparison with NC (0.96±0.12 μg/mL, p<0.001). Further agents afforded intermediary HYP-release outcomes.

Raman spectra of cross-linking formation are reported in Figure 4. All biomodification agents provided the emergence of a shoulder at approximately 1117 $cm^{-1}$and increase of 1235 cm^{-1} peak, thereby demonstrating collagen cross-linking.

DISCUSSION

The present investigation evaluated the contribution of lignin (from *Eucalyptus*) as biomodification agent in demineralized human dentin for the sake of improving biomechanical and biochemical properties. Moreover, it needs to be highlighted that this is the first study of lignin in adhesive dentistry. To compare with lignin, two previously proved biomodification agents (PAC from *Vitis vinifera* and cardanol from *Anacardium occidentale*) were included in study design. Present hypothesis needs to be rejected, once lignin in lower concentrations attained better overall outcomes than PAC and cardanol.

Lignin is an important biopolymer discarded by farming, biofuel and cellulose companies (Yoon et al. 2015). Besides, the lignin subproducts production in bio-refineries is underused to obtain aromatic biopolymers with antioxidant and antimicrobial capacity. Therefore, lignin is a promising raw material to produce applications with high added value (Mattinen et al. 2018). All plants possess high amount of lignin (~25% of biomass), becoming second most abundant polymer worldwide (Castilho-Almeida et al. 2013). Contrariwise to cellulose, lignin rarely presents monomeric structure (Ralph et al. 2004). However, its exact chemical structure is not fully known due to alterations undergone during wood processing. Lignin's mainframe is phenyl-propane, linked to benzene ring with variable number of hydroxyls (10%) and methoxyls (90%). Altogether, these functionalities comprise distinct phenols in an aromatic polymer complex of monomeric sub-unities, such as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Ralph et al. 2004; Yoon et al. 2015). Furthermore, structural complexity augments depending on internal cross-links, which differs in each plant specie (Castilho-Almeida et al. 2013). Based on the cross-linking ability of lignin at plant

cell-walls, the present investigation proposed to study its dentin biomodification properties since its antiviral, antineoplastic, anti-inflammatory and osteogenic activities have already been demonstrated (Sakagami et al. 2014; Sakagami and Tomomura et al. 2018).

Current trend in restorative dentistry addresses biomimetic recovery of shape and function of dental hard tissues (Bedran-Russo et al. 2014), highlighting biomimetic remineralization (Abuna et al. 2016) and biomodification of dentin with natural collagen cross-linkers (Bedran-Russo et al. 2014). The latter tends to augment reticulation of mature collagen matrix by non-enzymatic reactions, strengthening the tissue biomechanics and drastically dropping degradation (Vidal et al. 2016), using plant-derived polyphenols with low toxicity (Bedran-Russo et al. 2014). PACs are highlighted as condensed tannins with high protein affinity and antioxidant capacity, also investigated in functionalized biomaterials (He et al. 2011). Cross-linking does not depend only on hydroxyls, but on the entire molecular structure of PACs, including aromatic rings that trigger hydrophobic bonds (He et al. 2011; Vidal et al. 2016; Bedran-Russo et al. 2014).

Further biomodification agent used herein was cardanol, from cashew-nut shell liquid, obtained as a discard of cashew industry (Barreto et al. 2012; Lomonaco etal. 2017). Cardanol is a long carbon-chain (15 carbons) phenol with high hydrophobicity and several sites for organic synthesis (Lomonaco et al. 2017, Moreira et al. 2018). In dentistry, it showed optimal dentin desensitizing properties (Moreira et al. 2018) and ability to increase elastic modulus, diminishing biodegradation of demineralized dentin, without color alteration (Moreira et al. 2017).

The presence of all biomodification agents used as therapeutic primer after phosphoric-acid etching dentin and prior to the application of a two-step etch-and-rinse adhesive reduced initial bond strength (Table 1) , except LIG1, similarly to the results of Hass et al. (2016). This might occur due to steric obstruction to monomer polymerization in hybrid

layer, as confirmed by DC results (Table 1). PACs are oligomeric molecules, constituted majorly by sub-unities catechin, epicatechin and epigallocatechin. Indeed, the large molecular size of PAC may impair optimal penetration in collagen mesh, resulting in lower μTBS. Conversely, lignin possesses hetero-polymeric aromatic complex with three main units (Figure 1) characterized by hydroxyl and methoxyl functionalities (Agache and Popa 2006) that likely accomplish binding to collagen, which provided adequate μTBS, although it has large molecular size.

The aging protocol utilized for resin-dentin sticks was 1,000 thermal cycles, (Cotes, et al, 2016; El-Deeb et al, 2015). Perhaps, aging by simulated pulpal pressure (SPP) could afford excess of biomodification agent release from dentin interface, depending on molecular weight (Yoon et al. 2015). However, Perote et al. (2015) demonstrated both protocols (SPP and thermocycling) produce similar bond strength reduction. Most experimental groups maintained μTBS, thereby proving the bond stability thanks to dentin biomodification, which was demonstrated by the presence of Raman peaks 1117 and 1235 cm^{-1} referring to collagen cross-linking (Fig. 4) (Moreira et al. 2017; Vidal et al. 2016). Indeed, this improved dentin sealing as observed in confocal microscopy, mains achieved by PAC, CARD and LIG4 (Fig. 3).

SEM micrographs after aging (Fig. 2) depicted few deposits of water and voids within hybrid layers created with all biomodification agents, especially with lignin. The fact that CARD decreased μTBS after aging might be explained by its molecular structure comprising solely one hydroxyl able to create hydrogen bonds, thereby facilitating the leaching of biomodification agent over time. However, cardanol promoted noteworthy increase on elastic modulus, what is explained by its linear and low-molecular weight chemical structure (Fig. 1). Furthermore, the long and hydrophobic carbon chain may guarantee collagen protection during 4-week water storage (Moreira et al. 2017, Moreira et al. 2018).

Regarding LIG4, the excess of lignin provided additional cross-linking over time, what afforded μTBS increase. It was previously reported that cross-linking of a biomodification agent is dose-dependent (Seseogullari-Dirihan et al. 2017), corroborating for the explanation about this bond strength increase with LIG4. In negative control, the absence of biomodification agent demonstrated rapid interfacial degradation, mostly due to MMPs and resin-sparse unprotected collagen fibrils (Aydin et al. 2019), resulting in approximately 40% bond strength loss after thermocycling.

Proanthocyanidins from grape-seed extract (*Vitis vinifera*) is classified as condensed tannin with B-type interflavonoids links, depicting effectiveness in increasing the rigidity of demineralized dentin matrix higher than further non-galloylated compounds (Phansalkar, et al. 2015; Aydin et al. 2019). Nevertheless, this type of PACs are less stable in comparison with A-type ones (Xu et al. 2015), because galloyl-bonds are prone to hydrolysis of 3-O-gallate ester during aging, which might be accelerated by oxidation reactions (Xu et al. 2015). Indeed, this may explain the loss of activity of PAC in augmenting elastic modulus and providing mass gain, yielding intermediary release of HYP (Table 1).

Higher concentrations of lignin directly improved elastic modulus of dentin collagen (Table 1), in a similar way to what occurs in plant cell walls, where the bond between cellulose fibers and lignin is amplified by covalent bonds (Yoon et al. 2015). Nonetheless, lignin at 1% achieved better outcomes of mass change during water storage and least HYP release, thereby proving the efficacy in protecting demineralized dentin from degradation. Due to its irregular, branched and amorphous chemical structure, lignin in lower concentrations might be arranged and fill optimally the three-dimensional spaces among collagen fibrils. Moreover, its hydrophobic feature thanks to the aromatic rings and thermal stability due to intra-molecular cross-links provided stable collagen cross-linking for protection against hydrolysis and MMPs (Boteon et al. 2017).

In conclusion, lignin can realize cross-linking in demineralized dentin collage fibrils, improving the bond stability of composite restorations, and preventing collagen degradation, particularly when applied in 1% concentration, which did not affect the degree of conversion of adhesive. Future investigations should focus on further uses of lignin in restorative dentistry.

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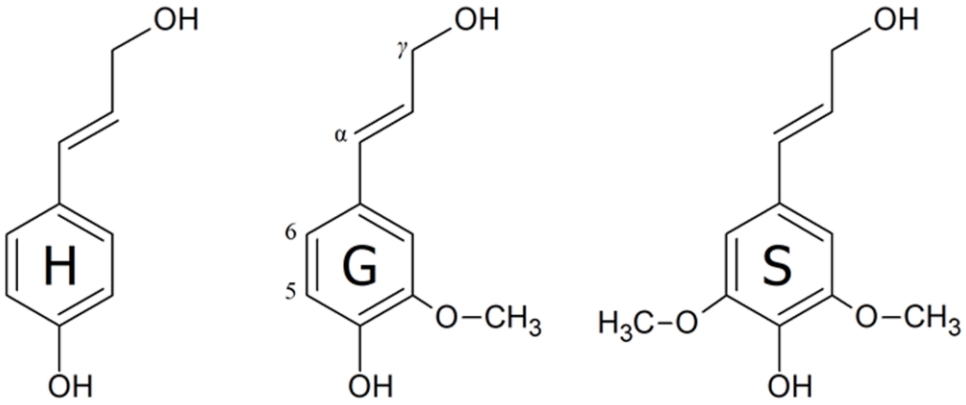
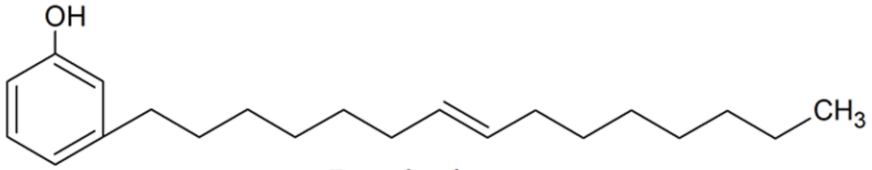
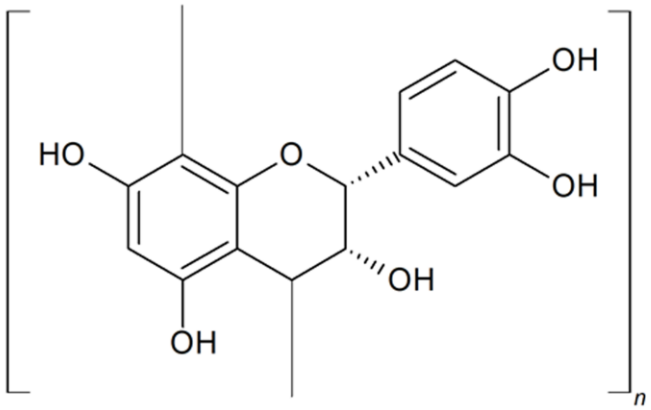
FIGURE, LEGENDS AND TABLES



A1. *p*-hydroxyphenyl A2. guaiacyl A3. syringyl

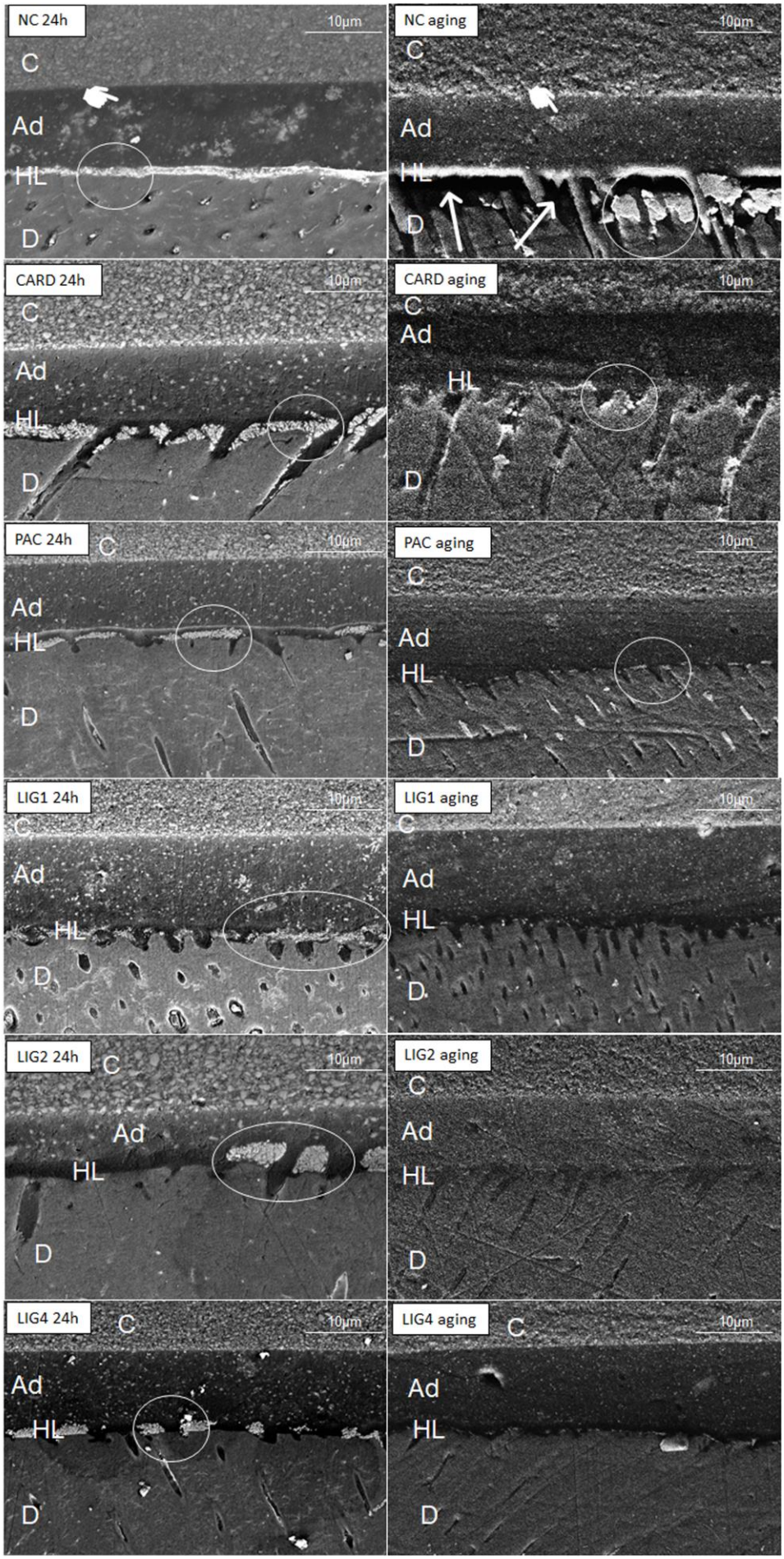


B. cardanol



C. condensed tannin (PAC)

Figure 1. Chemical structures of biomodification agents surveyed. A. Mono-lignols precursors of structural units forming the polymeric lignin molecule; B. Chemical structure of purified cardanol; C. Major structure of proanthocyanidins (PAC) from grape seed extract.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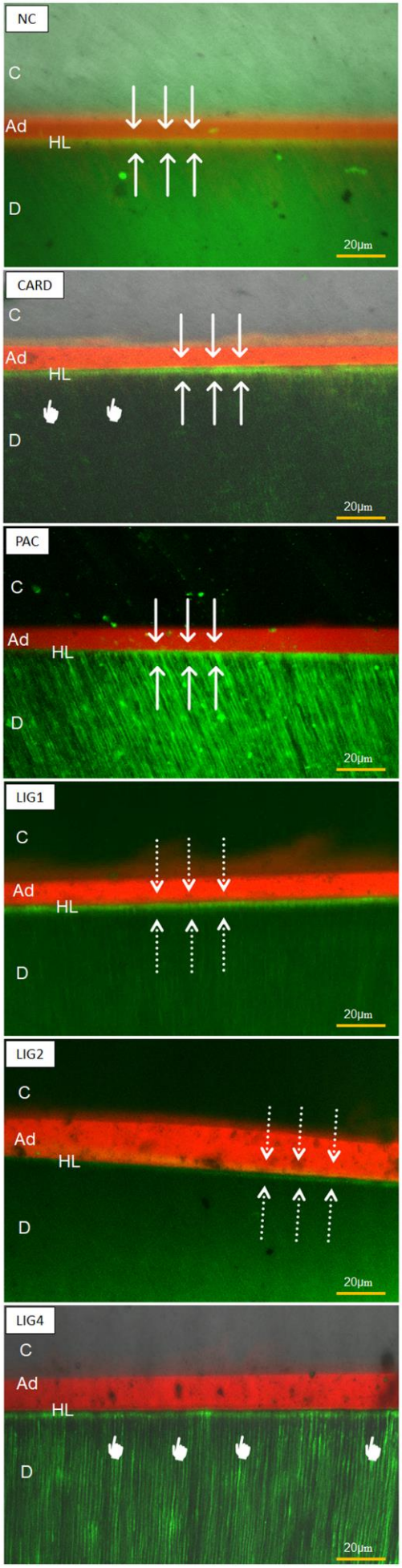
LIG2 aging
10μm
C
Ad
HL
D
LIG4 24h
10μm
C
Ad
HL
D
LIG4 aging
10μm
C
Ad
HL
D

Figure 2. SEM images of the bonding interface of resin-dentin speciments assayed 24h and after thermocycling aging. The pointers depict water-trees into the adhesive layer, arrows represent a fracture, and circles highlight silver infiltration into the hybrid layer. NC: Negative Control; CARD: Cardanol; PAC: Proanthocyanidin; LIG: Lignin; C: composite layer; Ad: adhesive layer; HL: hybrid layer; D, dentin. NC showed water-trees and striking silver uptake both in both 24h and after aging. CARD and PAC showed areas of silver uptake both in 24h and after aging. Water-trees were found only with PAC after aging. LIG1, LIG2 and LIG4 showed similar areas of silver uptake in HL, but very little nanoleakage after aging.


NC
C
Ad
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D
20μm
CARD
C
Ad
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20μm
PAC
C
Ad
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D
20μm
LIG1
C
Ad
HL
D
20μm
LIG2
C
Ad
HL
D
20μm
LIG4
C
Ad
HL
D
20μm

Figure 3. Confocal microscopy images representing the most common micropermeability outcomes at the bonding interfaces of resin-dentin specimens. The pointers represent the protected hybrid layer; the arrows represent large infiltration of fluorescein; dotted arrows represent little infiltration of hybrid layer. In general, most prominent micropermeability was observed in negative control and PAC-treated specimens, whilst fewest micropermeability was found specimens treated with 4% lignin. NC: Negative Control; CARD: Cardanol; PAC: Proanthocyanidin; LIG: Lignin; C: composite layer; Ad: adhesive layer; HL: hybrid layer; D: dentin.

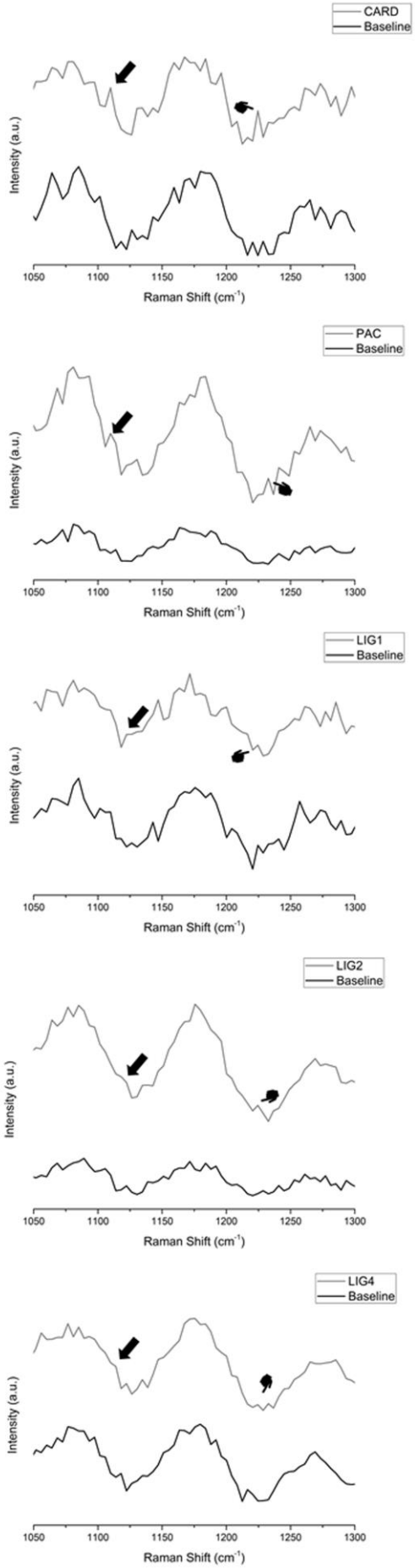
CARD
Baseline
Intensity (a.u.)
1050
1100
1150
1200
1250
1300
Raman Shift (cm-1)
PAC
Baseline
Intensity (a.u.)
Raman Shift (cm-1)
LIG1
Baseline
Intensity (a.u.)
Raman Shift (cm-1)
LIG2
Baseline
Intensity (a.u.)
Raman Shift (cm-1)
LIG4
Baseline
Intensity (a.u.)
Raman Shift (cm-1)

Figure 4. Vibrational Micro-Raman spectra of same specimens before (Baseline) and after 1 minute biomodification treatment. All agents induced emergence of shoulder at ∼1117 cm^{-1} (black arrow) and increase of Amide III peak at 1235 cm^{-1} (pointers) which demonstrates collagen crosslinking. CARD: Cardadol; PAC: Proanthocyanidin; LIG: Lignin.

Table 1. Means (standard deviations) of quantitative results of various experiments.

Groups	μTBS (MPa) [fracture mode A/CD/CC/M]		DC	Modulus of Elasticity			Mass Change (%)		HYP (μg/mL)
	Immediate	Aging	(%)	Baseline (MPa)	Treatment (MPa)	Variation (%)	After Biomodific.	4-weeks degradation	Not-demineralized 0.02 (0.01)^{D}
NC	51.2 (4.9)^{Aa} [90/2/3/5]	36.8 (7.2)^{ABb} [97/0/0/3]	75.7 (4.5)^{A}	6.8 (1.9)^{a}	4.1 (7.2)^{b}	-40.4 (23.2)^{C}	13.5 (6.7)^{C}	-34.4 (2.8)^{C}	0.96 (0.12)^{A}
LIG1	39.6 (3.3)^{ABa} [98/0/0/2]	35.7 (7.4)^{Ba} [92/0/0/8]	71.1 (4.3)^{AB}	3.6 (1.1)^{b}	4.3 (1.9)^{a}	22.8 (35.7)^{BC}	30.7 (8.6)^{A}	45.2 (13.2)^{A}	0.33 (0.01)^{C}
LIG2	38.1 (6.2)^{BCa} [98/0/0/2]	37.7 (2.6)^{ABa} [95/0/0/5]	64.1 (0.2)^{BC}	2.7 (0.8)^{b}	5.6 (2.2)^{a}	116.5 (74.5)^{AB}	25.7 (8.7)^{AB}	42.9 (19.1)^{A}	0.53 (0.01)^{B}
LIG4	37.5 (3.0)^{BCb} [99/0/0/1]	46.4 (5.6)^{Aa} [92/0/3/5]	64.1 (6.1)^{BC}	3.6 (1.2)^{b}	8.4 (3.7)^{a}	177.6 (63.8)^{A}	16.1 (11.5)^{BC}	27.3 (16.1)^{AB}	0.55 (0.09)^{B}
CARD	37.9 (4.0)^{BCa} [100/0/0/0]	31.2 (5.5)^{Bb} [97/2/0/1]	57.7 (2.3)^{C}	4.4 (1.0)^{b}	7.9 (3.9)^{a}	85.5 (96.7)^{AB}	21.4 (9,0)^{ABC}	20.3 (17.5)^{AB}	0.50 (0.07)^{BC}
PAC	30.3 (4.9)^{Ca} [100/0/0/0]	30.2 (4.0)^{Ba} [100/0/0/0]	56.8 (3.3)^{C}	7.4 (2.0)^{a}	8.6 (2.2)^{a}	17.1 (12.7)^{BC}	19.2 (5.4)^{ABC}	4.5 (3.0)^{B}	0.61 (0.06)^{B}

* Lowercase letters represent statistical differences in rows and uppercase letters in columns ($p<0.05$). NC: negative control; LIG: lignin; CARD: cardanol; PAC: proanthocyanidin. Fractures percentages - [adhesive/cohesive in dentin/cohesive in composite/mixed]

3.2 CAPÍTULO 2

Influence of collagen cross-linkers addition in phosphoric acid etchant on dentin biomodification and bonding of an etch-and-rinse adhesive

ABSTRACT

Purpose: To investigate the effects of natural collagen cross-linkers incorporation in phosphoric acid etchant on dentin biomodification, microtensile bond strength (μTBS) and nanoleakage (NL) of a two-step etch-and-rinse adhesive.

Methods: Experimental aqueous solution of 37% ortho-phosphoric acid were prepared with the addition of 2% biomodification agents: Lignin (LIG) from industrial paper production residue, Cardanol (CARD) from cashew-nut shell liquid, and Proanthocyanidin (PAC) from grape-seed extract. Negative control (NC) was acid solution without cross-linker whilst commercial control (CC) was Condac 37 gel (FGM). Dentin specimens were assayed by FTIR after 15s etching to detect collagen cross-linking. Extracted third molars were used for μTBS (n=7) and fracture mode analysis of Optibond S (Kerr), tested after 24h or 1,000 thermal cycles. NL was surveyed by SEM. Statistical analysis was performed with two-way ANOVA and Tukey's test ($p<0.05$).

Results: FTIR confirmed cross-linking for all agents. CC's μTBS was the highest (46.6±6.2 MPa), but reduced significantly after aging (35.7±5.2 MPa) ($p<0.001$). LIG (30.6±3.7 MPa) and CARD (28.3±1.8 MPa) attained similar μTBS which were stable after aging ($p>0.05$). Fracture mode was predominantly adhesive. At 24h, All groups showed presence of silver uptake in hybrid layer, except CARD. After aging, CARD- and LIG-treated specimens exhibited little amount of silver penetration. CC, PAC and NC showed gaps, great nanoleakage at hybrid layer and presence of water channels in adhesive layer.

Significance: Altogether, ortho-phosphoric acid incorporated with LIG and CARD promotes stable resin-dentin bond strength with minor nanoleakage after aging, thereby achieving therapeutic impact without additional clinical steps.

Keywords: Dentin. Lignin. Cardanol. Proanthocyanidin.

1. INTRODUCTION

The threshold research regarding the effectiveness of acid etching dental hard tissues for the sake of improving bonding was the investigation of Buonocore (1955) [1]. In that occasion, 85% phosphoric acid was employed to bond acrylic resin to enamel. Later, Nakabayashi (1982) [2] demonstrated the bonding mechanism relies on homogeneous infiltration and *in situ* polymerization of monomers in etched dentin, accomplishing, theoretically, a mechanically stable interface, the so-called hybrid layer.

Current dental bonding agents are classified as etch-and-rinse or self-etch [3]. The former addresses 30-40% phosphoric acid to demineralize (5-8 μm) dentin, exposing collagen mesh for infiltration of monomers to ensure the adhesion of composite restorations [4]. The exposed dentin matrix is composed by type-I collagen (90%) and non-collagenous proteins, such as proteoglycans and matrix metalloproteinases (MMPs) surrounding the tridimensional structure of collagen [5,6]. Acidic pH generated by bacteria metabolism may activate MMPs and induce release of cathepsins, which accelerate collagen degradation, even near hybrid layer [7]. In resin-dentin interface, rapid breakdown of unprotected collagen fibrils occurs mainly with etch-and-rinse adhesives, yielding restoration failures within the five initial years [4]. Water may also achieve polymer hydrolysis, thereby contributing for the bond shortcoming [8,9].

A feasible strategy to augment longevity of adhesive restorations is the biomodification of dentin substrate, promoting collagen cross-linking to increase biomechanical properties and diminishing biodegradation rates [10]. Such process reduces molecular mobility, inhibits MMPs and decreases water permeability [4,10,11]. The incorporation of biomodification agents in phosphoric acid etchant undertakes a rapid therapeutic procedure avoiding additional application of a primer. Proanthocyanidins (PAC)

from grape-seed extract (*Vitis vinifera*) and chlorhexidine (CHX) added to the acid etchant previously demonstrated reliable outcomes on dentin bond stability [12-16].

Cardanol (CARD) from cashew nut shell liquid (*Anacardium occidentale*) is a product of discard from nut industry, which recently demonstrated optimal collagen cross-linking ability [17] without staining the dentin [18]. Also, lignin (LIG) is a natural polymer from plant cell walls, responsible for increase their rigidity [19,20] with chemical structure rich in phenols (Fig. 1) potentially able to attain collagen cross-linking [10]. Both agents (CARD and LIG) were never investigated in Dentistry incorporated in phosphoric acid etchant in the attempt to augment the longevity of composite restorations.

Therefore, the objective of this study was to evaluate the biomodification capacity of LIG and CARD in human dentin added to phosphoric acid etchant as well as the dentin bonding of an etch-and-rinse adhesive. Hypotheses are 1) addition of all biomodification agents in experimental acids achieves collagen cross-linking and 2) the incorporation of biomodification agents to etchant affords bond strength stability after aging, due to particular chemical structures with phenolic compounds (Fig.1)

2. MATERIALS AND METHODS

2.1. Specimens' preparation

Thirty five sound human third molars were extracted for reasons apart from this investigation were used after approval of Institutional Ethics Committee (protocol 011133/2018). They were stored in 0.1% thymol solution and used within two months after extraction. Flat middle dentin surfaces were obtained after removing the occlusal enamel by cutting with a diamond saw adapted in a cutting machine (Cutmaster, São Paulo, Brazil).

Exposed dentin surfaces were wet-abraded with 320-grit SiC polishing papers to obtain standardized smear layers.

2.2. Experimental design

CARD was acquired from cashew nut shell liquid, which was gently donated by Amendoas do Brasil LTDA (Fortaleza, Brazil) separated by the methodology described by Lomonaco et al. (2013) [21]. Grape-seed extract (*Vitis vinifera*, >90% PAC, Meganatural Gold, Madera, USA) was used to attain PAC. Lignin was donated from paper industry Suzano Papel e Celulose SA (Limeira, Brazil) and used without further purification. Acid etchant solutions were prepared by adding 2wt% biomodification agents [14] in 37% ortho-phosphoric acid solution of ethanol:water (at 1:1 ratio). Solutions' pHs were standardized (<1.0) prior to using [16]. The experimental 37% phosphoric acid solution without biomodification agent was employed as negative control (NC). Commercial control (CC) was Condac 37% phosphoric acid gel (FGM Dental Products, Joinville, Brazil). Purification and characterization of cardanol and lignin are described in detail in supplemental material.

2.3. FTIR spectroscopy

The dentin surfaces were investigated after 15s application of each one of the five acids and 30s rinsing with distilled water. Spectroscopic analysis was attained by Fourier-transform infrared spectroscopy (Spectrum Frontier, Perkin Elmer Corp., Norwalk, USA) with 4cm^{-1} resolution, 32 scans in transmittance mode. The spectra range was 1800-1000 cm^{-1} to survey peaks and shoulders at 1633 cm^{-1} (amide I), 1544 cm^{-1} (amide II), 1450 cm^{-1} (CH_2 bending) and 1235 cm^{-1} (amide III) assigned to dentin collagen cross-linking [22,23]. The analysis was realized in triplicate.

2.4. Restorative procedures

The dentin surfaces were etched with experimental or commercial acids for 15s and rinsed with distilled water for 30s. Following, the two-step etch-and-rinse adhesive Optibond S (Kerr, Orange, USA) was actively applied for 20s, slightly air-thinned for 3s and light-cured for 20s with LED unit DB-685 (1100 mW/cm2, Dabi Atlante, Ribeirão Preto, Brazil). Composite build-ups were constructed with 1mm-thick layers of micro-hybrid composite Opallis (FGM), which were individually light-cured for 20s [13]. Bonded specimens were stored in distilled water for 24h.

2.5. Microtensile bond strength (μTBS) and Fracture mode

Bonded teeth (n=7) were longitudinally cut in 1mm-thick slices and turned 90º to perform further cuts and obtain resin-dentin sticks with approximately 1mm² cross-sectional area. Half of sticks per tooth were tested immediately and further half was tested after 1,000 thermal cycles (30s in 5ºC and 30s in 55ºC with 10s interval) (Huber-Mechatronik TC45SD, SD Mechatronik, Feldkirchen-Westerham, Germany) [24, 25]. Microtensile test was performed by attaching the resin-dentin sticks in Geraldeli's jigs with cyanoacrylate glue, adapted in a microtensile machine (OM-100, Odeme, Luzerna, Brazil) and tested in tensile until fracture with 0.5mm/min crosshead speed and 500N load cell. Before testing, each stick was measured with digital calliper (0.01 mm precision) to obtain the bond strength in megapascals (MPa) [13]. Pre-test fractures were not frequent (less than 3 per group) and were included as 0 MPa.

All fractured sticks were examined in a stereomicroscope (60x magnification, Stereo-zoom S8, Leica, Heidelberg, Germany) in order to identify the failure modes, which were

classified as adhesive, cohesive in dentin, cohesive in composite or mixed (partially adhesive and cohesive).

2.6. Nanoleakage assessment

Two resin-dentin sticks from each subgroup were surveyed for interfacial silver nanoleakage following the protocol of Tay et al. (2002) [25], using 50% ammoniacal silver nitrate solution. Briefly, specimens were immersed in the tracer silver solution for 24h protected from light, washed with distilled water and immersed in photodeveloping solution for 8h under fluorescent light. Afterwards, they were embedded in epoxy resin and polished with SiC papers up to 4000-grit and 1-µm diamond paste (Buehler, Coventry, UK) in polishing clothes [26]. They were cleaned for 5min in ultrasonic bath after each polishing step and dehydrated for 24h in an incubator with silica gel at 37ºC. They were sputter coated with gold and observed in scanning electron microscopy (Quanta FEG, FEI, Amsterdam, Netherlands) in backscattered electron mode with 10kV and 10mm working distance.

2.7. Statistical analysis

Initially, data from µTBS (MPa) were subjected to Shapiro-Wilk test to evaluate the normal distribution. Then, they were subjected to two-way ANOVA (five levels of acids, and two levels of aging) and Tukey´s post-hoc test (α=0.05).

3. RESULTS

FTIR spectra of etched specimens are shown in Figure 2. All biomodification agents attained increase of aforementioned peaks (1633 cm^{-1}, 1544 cm^{-1}, 1450 cm^{-1} and 1235 cm^{-1}), with notable enlarging of peak 1544 cm^{-1} only with specimens conditioned with acids containing PAC, CARD and LIG, thereby demonstrating collagen cross-linking. Yet, shoulders at ~1400 cm^{-1} [22] and ~1080 cm^{-1} [23] emerged with the presence of all three biomodification agents.

The outcomes of μTBS are summarized in Figure 3. Commercial control attained superior bond strength initially, but with significant drop after aging ($p<0.001$). Such decrease occurred also with negative control ($p=0.02$). LIG (30.6±3.7 MPa) and CARD (28.3±1.8 MPa) achieved similar initial μTBS ($p=0.881$), which remained stable and resembling after aging (30.9±4.2 and 28.9±4.3 MPa respectively; $p=0.935$). PAC-containing acid yielded the lowest initial bond strength (17.7±0.9 MPa) with statistical drop after aging (9.9±2.3 MPa; $p=0.003$). The spreading of failure patterns is presented in Figure 4. Adhesive fractures were predominant in most groups, particularly after aging. However, with commercial control acid at 24h, cohesive in dentin was slightly higher than adhesive mode.

Nanoleakage representative micrographs are depicted in Figure 5. Striking silver infiltration was observed in interfaces of commercial control (at 24h and after aging), LIG (24h) and negative control (at 24h and after aging). Interfacial gaps were frequent in negative control (at 24h and after aging), and after aging with commercial control and with PAC-containing acid. LIG after aging and Cardanol demonstrated the highest resistance against silver impregnation, representing resin-dentin bonds almost devoid of defects and water.

4. DISCUSSION

The incorporation of collagen cross-linkers in phosphoric acid etchant is more feasible

than their application as a therapeutic primer, which adds a further clinical step to the procedure. Indeed, the use of etch-and-rinse adhesives following dentin phosphoric acid etching is still widely employed. However, stable dentin bonds are accomplished especially by adding a biomodification agent to the acid etchant [12,13]. The thermocycling aging protocol addressed herein was similar to the regimen of Cotes et al (2016) and El-Deeb et al (2015) [24, 25], which demonstrated μTBS reduction resembling to simulated pulpal pressure challenge. As expected, experimental water/ethanol solutions of phosphoric acids achieved lower substrate etching than commercial acid gel. However, experimental acid containing biomodification agents attained collagen cross-linking (Fig. 2), resulting in acceptance of first hypothesis. Nevertheless, bonding stability was obtained only with cardanol and lignin, but not with PAC. Therefore, the second hypothesis is accepted for CARD and LIG, but rejected for PAC.

Protection against degradation of demineralized dentin collagen, in presence of water, is currently clinically undertaken by means of MMP inhibitors in order to augment longevity of resin-dentin bonds [28]. Nonetheless, some investigations [12,14] recently demonstrated, by using μTBS and nanoleakage experiments, dentin bond stability of two-step etch-and-rinse adhesives when acid etchant was doped with collagen cross-linking agents, even after five years aging in distilled water [13]. Their findings corroborate with the present outcomes, once both acids free of biomodification agents (negative and commercial controls) did not protect hybrid layer (Fig. 5) and suffered bond strength decrease (Fig. 3) after aging, with consequent increase of adhesive failure percentage (Fig. 4).

The presence of CARD and LIG in acid etchant promoted hybrid layer protection against hydrolysis and MMPs, as they maintained the bond strength stable with relatively high adhesive fracture pattern (Fig. 4). With these particular agents, few silver uptakes were observed in electron microscopies (Fig. 5). Concerning cardanol, its hydrophobic 15-carbon

chain may impair the breakdown of the molecule in very acidic pH (<1). Nevertheless, the long time in acidic solution may likely achieve the breaking of double bond from alkene in the carbon chain. This might allow possible covalent bonds with collagen amino acids in addition to oligomerization of cardanol molecules, attaining higher degree of reticulation [29]. Moreover, cardanol is a relatively small molecule, which may rapidly penetrate around collagen fibrils [18,30,31] in the short application time used (15s). At last, the hydroxyl in cardanol molecular may enhance collagen cross-linking by the formation of hydrogen bonds [17,18].

Due to its hydrophobic nature, high amount of aromatic rings and molecule complexity based on p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Fig. 1), lignin possess high resistance to acidic environments [32,33]. Besides, the irregular, branched and amorphous architecture of lignin with tridimensional bonds may accomplish a suitable arrangement between collagen fibrils, thereby protecting collagen mesh similarly to plant cell walls [20]. This explains optimal outcomes of lignin-containing acid in preserving bond strength (Fig. 3) and increasing the water resistance of resin-dentin interfaces (Fig. 5).

In the case of acid containing PAC from grape-seed extract, the initial bond strength was the lowest among all acids, with a further significant drop after aging (Fig. 3). Suitable explanations for this are the short application time (15s), much lower than periods employed previously in literature, such as 10 minutes [11] and 1 hour [34,35]. A similar negative result was found in the study of Moreira (2017) [18], which observed severe collagen degradation for specimens treated with PAC, even with 1 minute application as a primer. As well as, in study of two-year clinical evaluation of cervical restorations with PAC added to two-step etch-and-rinse adhesive [36]. Yet, the acidic media may promote the breakdown of B-type interflavane links of PAC [31,35,37], which is a condensed tannin. Such bonds are less stable in low pHs, thereby triggering the breaking of 3-O-gallate esters [38]. Furthermore, the

phosphoric acid concentration in solutions was based on that of commercial control (37%) for standardization purposes. However, this high acid content may lead to non-synchronized penetration of PACs, which are formed by dimeric, trimeric and oligomeric structures possessing different molecular weights. Indeed, this acidic environment with water and ethanol as solvents may afford formation of micelles, which certainly contributed negatively to the infiltration of PACs in collagen mesh at intertubular dentin.

With control acids and PAC-containing one, demineralized collagen fibrils remained unprotected and prone to the activity of MMPs and other enzymes [16]. Liu and collaborators (2014) [16] suggests that the incorporation of PAC from grape-seed extract in phosphoric acid should be performed with acid concentrations lower than 20%. Another explanation for the lack of benefits with PAC-containing acid relies on the concentration of PAC. We standardized biomodification agent concentration in 2% according to Loguercio (2017) [14], whilst most investigations employ PAC in 6.5% [11,18,34,35,39]. Indeed, this reduction in PAC concentration may hinder its efficacy as biomodification agent.

Cardanol and lignin are products with sustainability appeal, once they are industrial wastes. Annual production of cashew nut shell liquid (with cardanol as major component) is 1,000,000 tonnes [29]. In the case of lignin, the global production might be much higher, as it is obtained from industrial production of paper and cellulose. Based on the present study, further useful applications are demonstrated for both compounds, thereby generating a finality with higher added value.

5. CONCLUSION

Phosphoric acid etchants incorporated with lignin and cardanol provide stable dentin bonds with low nanoleakage after aging and optimal collagen cross-linking, highlighting feasible usage in restorative dentistry.

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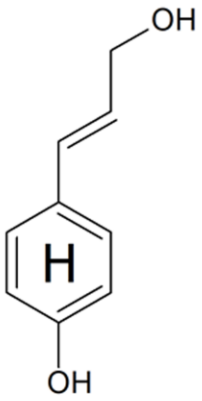
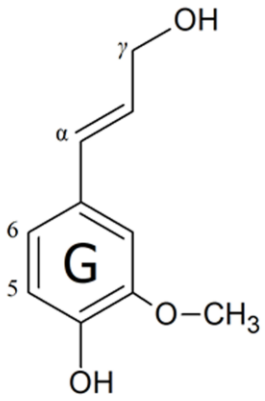
FIGURES, LEGENDS AND TABLES



A1. *p*-hydroxyphenyl



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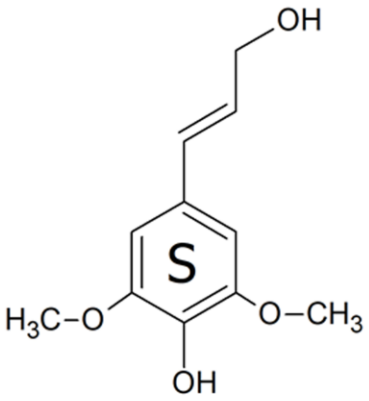
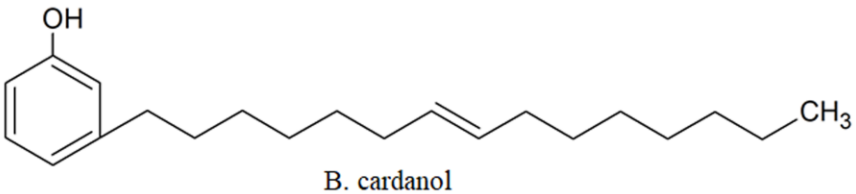
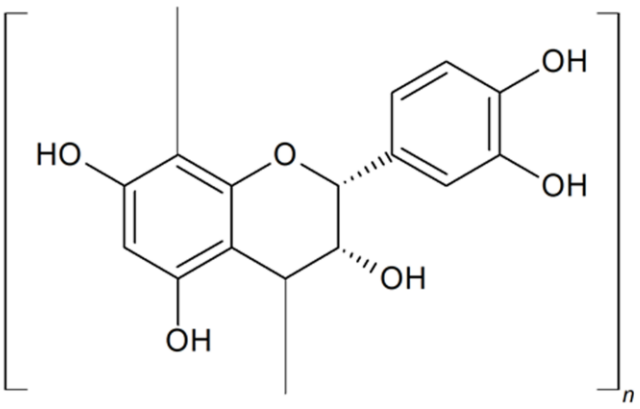


A3. syringyl



B. cardanol



C. condensed tannin (PAC)

Figure 1. Chemical structures of biomodification agents surveyed. A: Monolignols precursors of structural units forming the polymeric lignin molecule; B: Chemical structure of purified cardanol; C: Major structure of proanthocyanidins monomeric unit from grape seed extract.

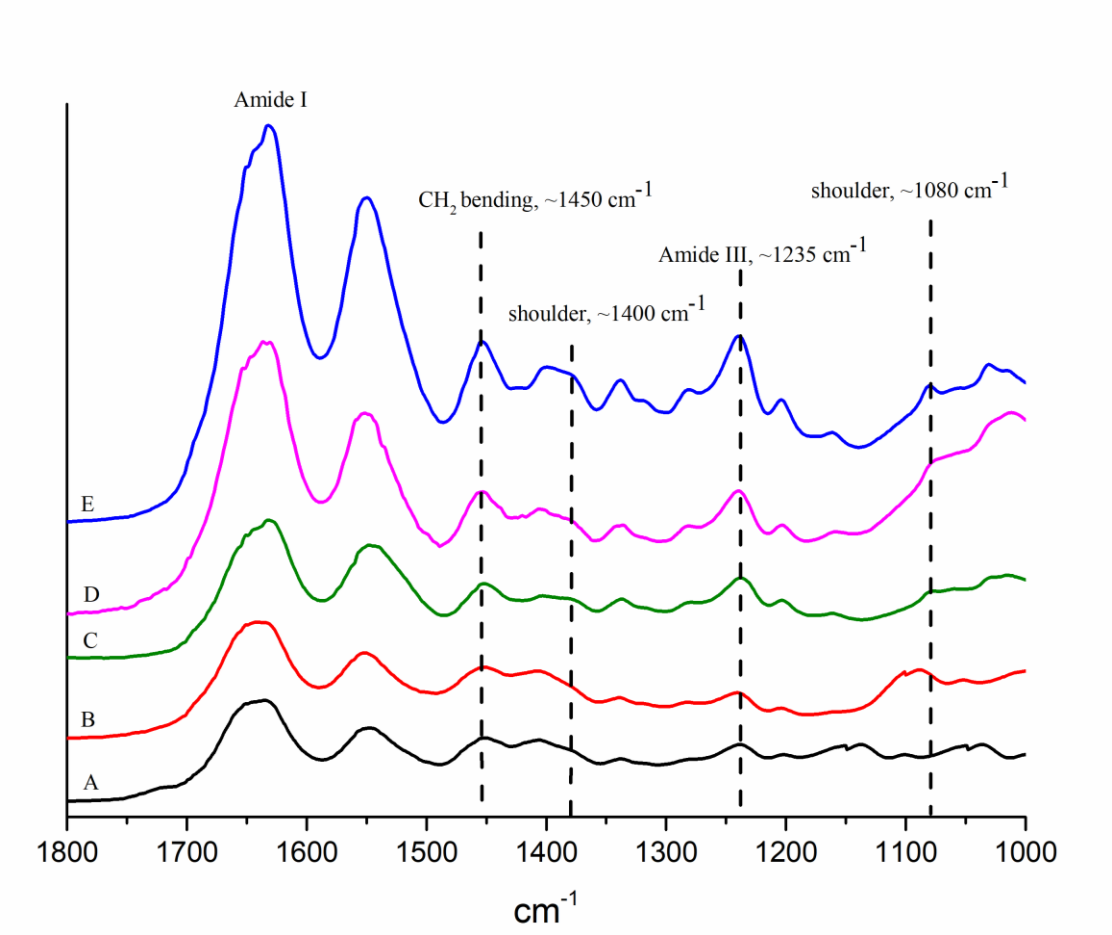


Figure 2. FTIR spectra of dentin specimens conditioned with different acids. Shoulders at ~1400 cm^{-1} and ~1080 cm^{-1} increased only with three biomodification agents. A: Dentin etched with commercial control acid; B: Dentin conditioned with negative control acid; C: Specimens etched with lignin-containing acid; D: Specimens conditioned with PAC-containing acid; E: Dentin substrate treated with cardanol-containing phosphoric acid.

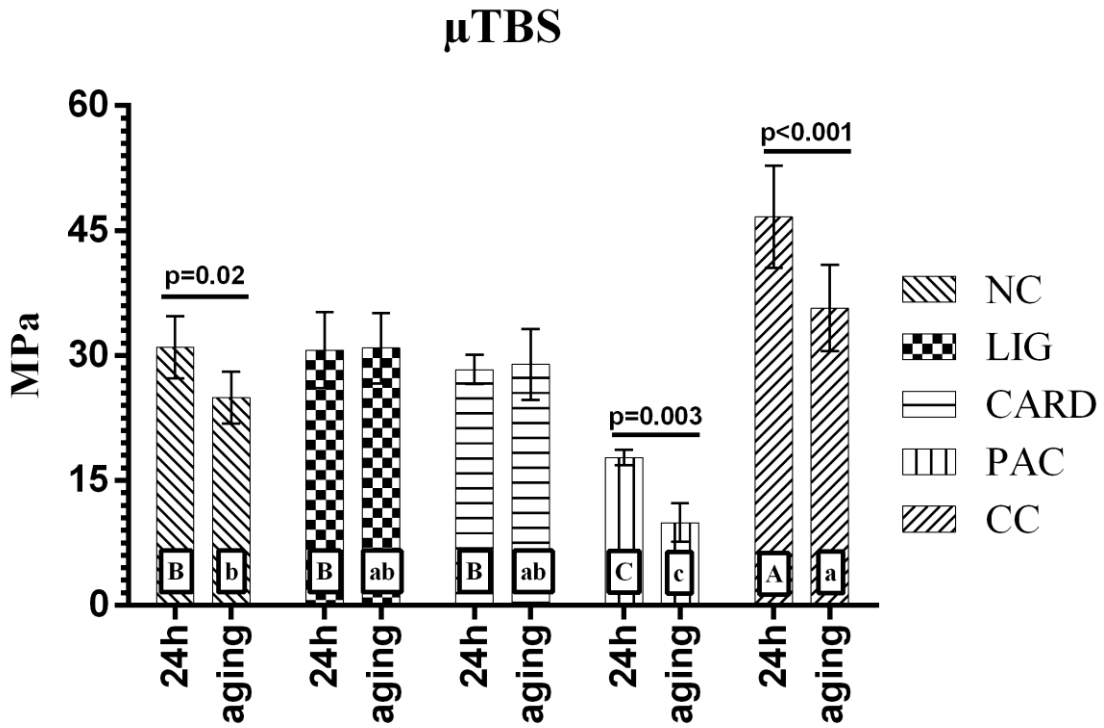


Figure 3. Means and standard deviations of resin–dentin microtensile bond strength (μTBS) of all groups. Capital letters represent statistical differences in 24h, whilst lowercase letters depict significant differences after aging. The black lines with p-values on the top indicate differences in the same group before and after aging. NC: Negative Control; CARD: Cardanol; PAC: Proanthocyanidin; LIG: Lignin; CC: Commercial Control.

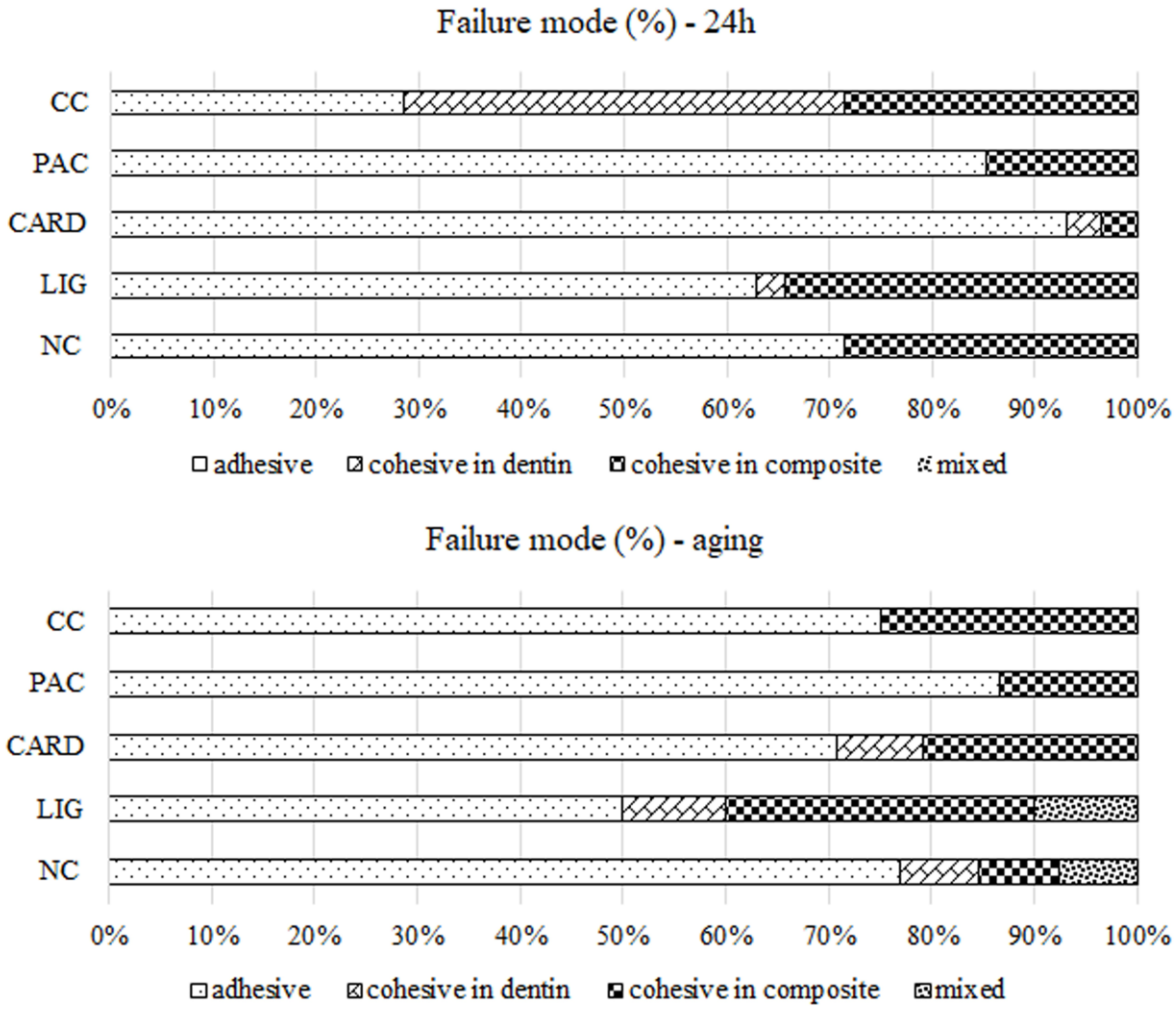


Figure 4. Failure mode percentages obtained in all groups after microtensile test. NC: Negative Control; CARD: Cardanol; PAC: Proanthocyanidin; LIG: Lignin; CC: Commercial Control.

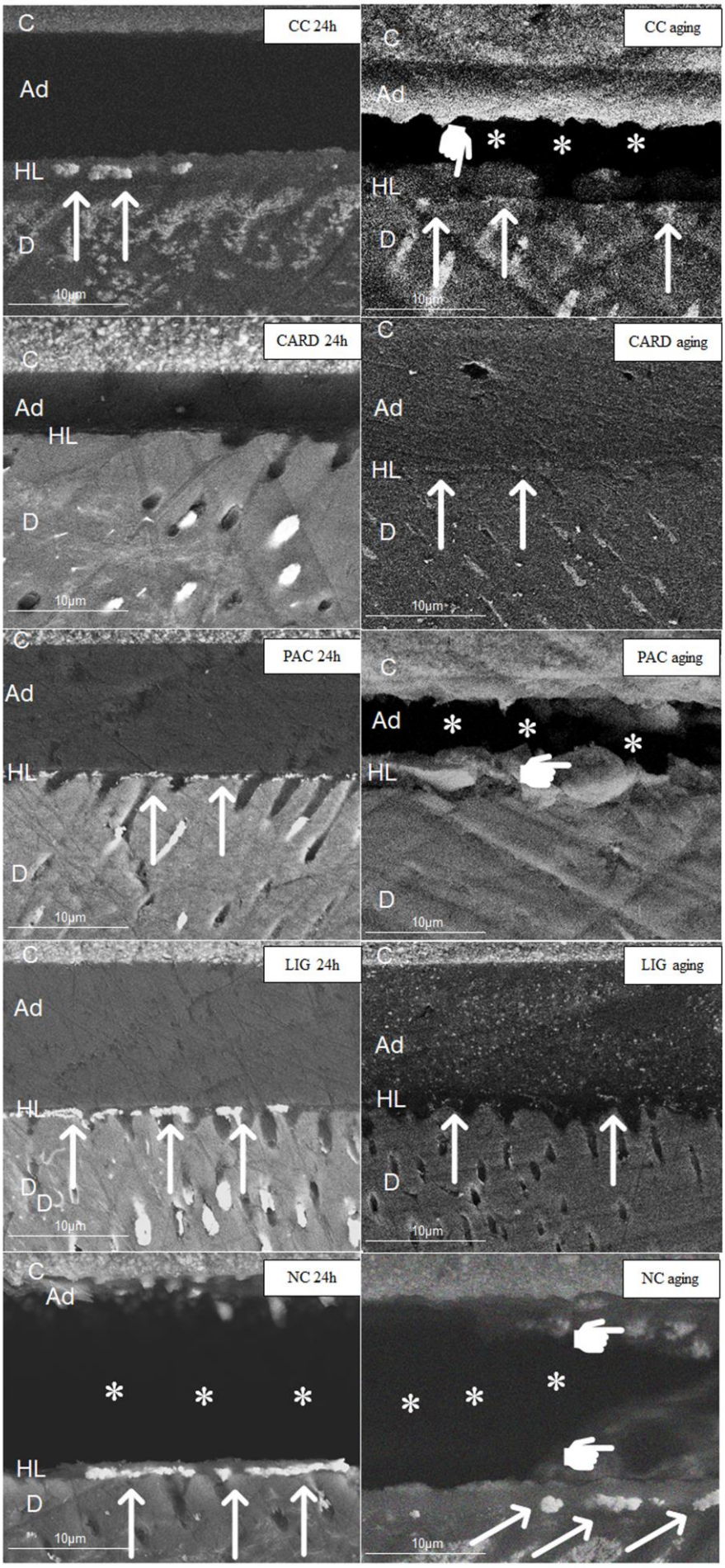

CC 24h
CC aging
CARD 24h
CARD aging
PAC 24h
PAC aging
LIG 24h
LIG aging
NC 24h
NC aging
C
Ad
HL
D
10μm

Figure 5. SEM images of the bonded interfaces assayed at 24h and after aging. The pointers represent water-trees into the adhesive layer; arrows highlight silver infiltration into the hybrid layer, and asterisks indicate gaps within the adhesive layer. In 24h, all groups showed presence of silver in the HL, except CARD. After aging, CARD and LIG revealed little silver uptake. CC, PAC and NC depicted notable silver impregnation in HL, water-trees in the adhesive layers as well as interfacial gaps. NC: Negative Control; CARD: Cardanol; PAC: Proanthocyanidin; LIG: Lignin; CC: Commercial Control; C: composite resin; Ad: adhesive layer; HL: hybrid layer; D: dentin.

4 CONCLUSÃO GERAL

A lignina foi capaz de agir como agente biomodificador na dentina humana desmineralizada, pois melhorou as propriedades de proteção do colágeno, preveniu a sua degradação, além aumentar resistência de união adesiva quando aplicada na forma de pré-tratamento de restauração de resina composta ou incorporada no ácido condicionante experimental, na técnica de adesivo convencional de dois passos.

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ANEXO - COMITÊ DE ÉTICA

UFC - UNIVERSIDADE FEDERAL DO CEARÁ /

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: UMA NOVA TECNOLOGIA PARA O REAPROVEITAMENTO DA BIOMASSA RESIDUAL DA INDÚSTRIA DO PAPEL

Pesquisador: DIEGO MARTINS DE PAULA

Área Temática:

Versão: 2

CAAE: 83249618.0.0000.5054

Instituição Proponente: Departamento de Odontologia Restauradora

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.544.896

Apresentação do Projeto:

Estratégias de biomodificação da dentina com o intuito de melhorar as propriedades mecânicas da camada híbrida e de proteger as fibrilas colágenas da degradação tem ganhado cada vez mais destaque. O presente trabalho tem como objetivo avaliar a capacidade da lignina agir como biomodificador crosslinking na dentina humana sadia. Para isso, serão fabricados primers contendo lignina (1, 2 e 4%), cardanol 2% e proantocianidina 6,5% aplicados por 1 minuto. A água destilada será utilizada como grupo controle negativo. Serão realizados os testes quantitativos de resistência de união à microtração (n=5) imediato e envelhecido por 6 meses, módulo de elasticidade (n=3) e hidroxiprolina (n=3), além da nanoinfiltração (n=6), micropermeabilidade (n=3) e espectroscopia Micro-Raman (n=3) como testes qualitativos. Os dados serão submetidos à análise estatística apropriada para cada experimento.

Objetivo da Pesquisa:

Objetivo Primário:

O presente trabalho tem como objetivo avaliar a capacidade da lignina agir como biomodificador crosslinking na dentina humana sadia.

Objetivo Secundário:

Avaliar capacidade da lignina realizar crosslinking na dentina sadia aplicada como pré-tratamento das restaurações de resina composta; Comparar a lignina com outros agentes naturais já

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Telefone: (85)3366-8344 **E-mail:** comepe@ufc.br

comprovados na literatura.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos estão inerentes à cirurgia de para remoção dos dentes que serão doados por motivos outros à pesquisa. No entanto, a pesquisa possui riscos mínimos por se tratar de pesquisa laboratorial.

Benefícios:

Os benefícios serão a possibilidade de melhorar a longevidade das restaurações de resina composta, bem como fornecer uma uma finalidade para o subproduto da queima do papel.

Comentários e Considerações sobre a Pesquisa:

Trata-se de uma pesquisa laboratorial de utilização de um subproduto da fabricação do papel para melhoras a resistência de restaurações de resina composta.

Considerações sobre os Termos de apresentação obrigatória:

Os termos de apresentação obrigatória foram devidamente apresentados. O pesquisador refez o que foi solicitado.

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

Não se aplica.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1060152.pdf	01/03/2018 16:15:35		Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	01/03/2018 16:15:12	DIEGO MARTINS DE PAULA	Aceito
Brochura Pesquisa	Brochura.docx	01/03/2018 16:09:56	DIEGO MARTINS DE PAULA	Aceito
Declaração de Instituição e Infraestrutura	AutorizacaoInstitucionalInfraestrutura.jpg	16/02/2018 16:24:01	DIEGO MARTINS DE PAULA	Aceito
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UFC - UNIVERSIDADE FEDERAL DO CEARÁ /

Continuação do Parecer: 2.544.896

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TCLE / Termos de Assentimento / Justificativa de Ausência	IsencaoDoTCLE.pdf	29/01/2018 18:11:01	DIEGO MARTINS DE PAULA	Aceito
Declaração de Pesquisadores	DeclaracaoDeConcordancia.pdf	29/01/2018 14:26:57	DIEGO MARTINS DE PAULA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

FORTALEZA, 15 de Março de 2018

Assinado por:
FERNANDO ANTONIO FROTA BEZERRA
(Coordenador)

Endereço: Rua Cel. Nunes de Melo, 1000
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APÊNDICE (Capítulos 1 e 2)

SUPPLEMENTAL APENDIX

1. Lignin purification and characterization

1.1. Materials

Glacial acetic acid, ethanol, DMSO-d_6 (99.96 %), chromium (III) acetylacetonate, cyclohexanol, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP), and THF (HPLC-grade) were supplied Sigma-Aldrich. Kraft Lignin (KL) was gently supplied by Suzano Papel e Celulose S/A (Brazil).

1.2. Organic solvent fractionation

Kraft Lignin (**KL)** (1g) was solubilized in 10 mL of ethanol and continuously stirred at room temperature during 30 minutes. The soluble fractions were concentrated under reduced pressure, yielding the lignin ethanol-soluble fraction named **KL-EtOH**. The obtained fraction was characterized by gel permeation chromatography (GPC), ^{31}P NMR and ^{1}H-^{13}C HSQC NMR.

1.3. Analytical techniques

^{1}H-^{13}C Heteronuclear single quantum coherence (HSQC) NMR spectra were recorded at 25 ^{o}C on a Bruker Avance DPX 300 spectrometer (operating at 300 MHz for ^{1}H nucleus), equipped with a 5-mm One Probe with z-gradient coils. Lignin samples (30 mg) were solubilized in 500 µL of DMSO-d_6. The central solvent cross-peak was used as internal reference (DMSO δ_H/δ_C 2.49/39.5).

GPC analyses were performed in a Shimadzu LC-20AD (Kyoto, Japan) at 40 ^{o}C using a setup comprising two analytical GPC columns in series (Phenogel 5µ 50Å and Phenogel 5µ 10^3Å, 7.38 mm x 300 mm, Phenomenex, Torrance, CA, USA) and HPLC-grade THF as mobile phase. The samples were monitored by UV-Vis detector (Shimadzu SPD-M20A) at

280 nm. Lignin samples (2 mg) were dissolved in 2 mL HPLC-grade THF and then were filtered using a 0.22 µm PTFE filter. Thus, 20 µL of filtered solution was injected into GPC system at a flow rate of 0.35 mL min^{-1}. Standard calibration was performed with polystyrene standards PSS (M_w range 162 – 1.3 x 10^5 g mol^{-1}).

2.5.5. ^{31}P NMR Spectroscopy

In order to determine the amount of aliphatic, phenolic and carboxylic acids hydroxyl groups present in lignin, ^{31}P NMR experiments were performed based on the methodology described by Granata and Argyropoulos with slight modifications. (Granata et al, 1995) Before sample preparation, all lignins were dried at 105 ˚C during 5 hours. Then, samples were weighted (30 mg) and dissolved in 500 µL of solvent mixture (C_5H_5N:$CDCl_3$, 1.6/1 v/v ratio). To the resultant solution, 100 µL of chromium (III) acetylacetonate solution (5.0 mg mL^{-1}) and 100 µL of cyclohexanol solution (10.85 mg mL^{-1}) were added. Finally, 100 µL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP) was added to the mixture, followed to the addition of 250 µL of solvent mixture in order to reach the mark of 1 mL of solution. The flask was tightly closed and shaken to ensure the complete dissolution of all components. The spectra were recorded on Bruker Avance DPX500 spectrometer (operating at 202.4 MHz for ^{31}P nucleus). All chemical shifts reported are related to the hydrolysis reaction of Cl-TMDP, which produces a sharp signal in C_5H_5N:$CDCl_3$ at 132.2 ppm. Quantitative analysis using cyclohexanol as internal standard was performed based on previous literature reports (Avelino et al, 2018). As a manner to establish a pattern of integration, the signals were integrated according to the following ranges of chemical shifts: internal standard (145.39 – 144.97 ppm), aliphatic-OH (150 – 145.50 ppm), C_5-substituted-OH (144.5 – 141.2 ppm), guaiacyl-OH (141 – 138.50 ppm), *p*-hydroxyphenyl-OH (138.4 – 137.20 ppm) and COOH-OH (136.5 – 133.34 ppm).

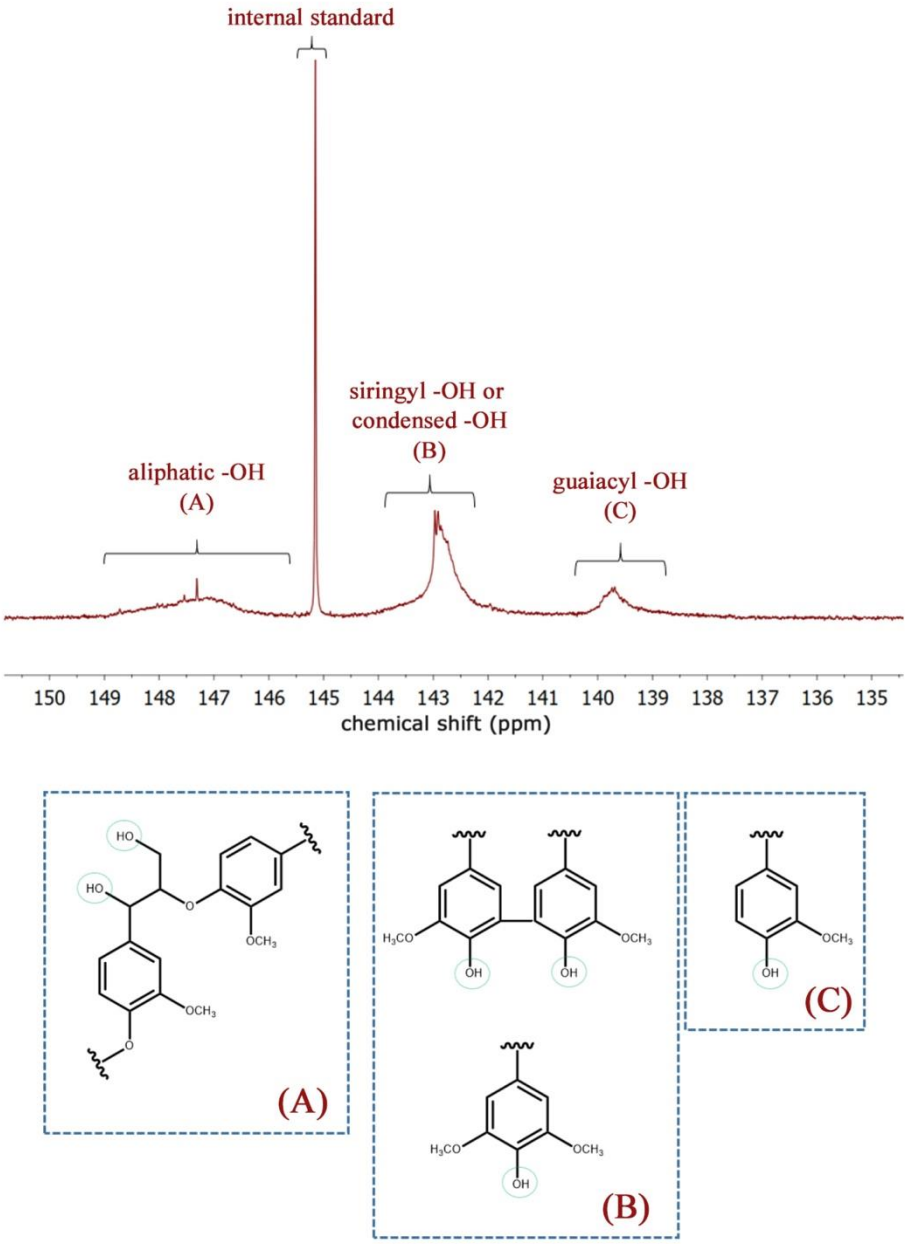

Figure 1. Quantitative ^{31}P NMR spectra of ethanol-soluble Kraft Lignin.

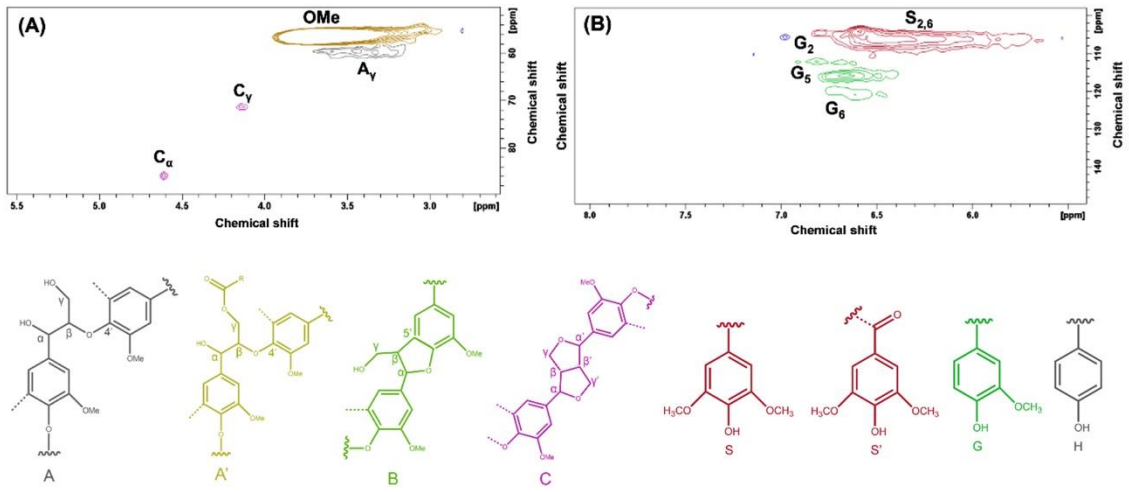

Figure 2. Aromatic (A) and oxygenated aliphatic (B) regions in ^{1}H-^{13}C HSQC NMR spectra of ethanol soluble Kraft Lignin.

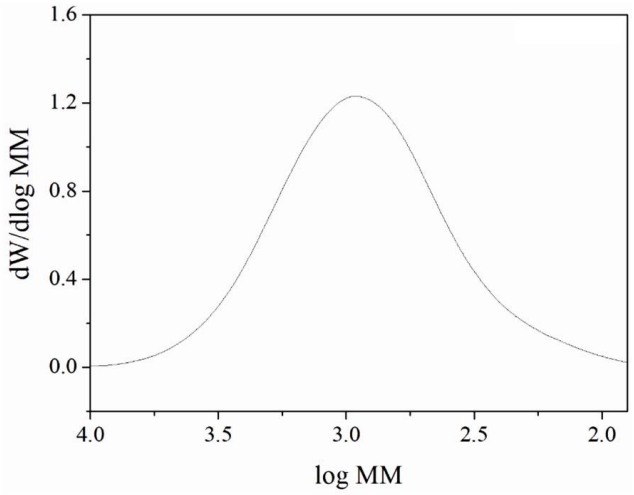

Figure 3. Differential molecular weight distribution of ethanol soluble Kraft Lignin obtained by GPC.

Table 1. Summarized results obtained from NMR and GPC analyses of ethanol soluble Kraft Lignin.

Index	Value
OH aliphatic content (mmol g^{-1})	0,70
Condensed or siringyl –OH (mmol g^{-1})	1,33
Guaiacyl-OH (mmol g^{-1})	0,42
OH phenolic content (mmol g^{-1})	1,75
Total OH groups' content (mmol g^{-1})	2,45
M_w (g mol^{-1})	1093
M_n(g mol^{-1})	656
M_w/M_n	1,67

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