The Effect of Cocoa Pod Husk and Green Tea Extract on SMAD3 and FGF2 Expressions in Exposed Dental Pulp

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Abstract

Direct pulp capping employing calcium hydroxide has been used to maintain the pulp's vitality and health and encourage the pulp cells to establish reparative dentin. It has been recommended to use calcium hydroxide as a material of direct pulp capping due to its beneficial properties. However, calcium hydroxide also has several weaknesses. Cocoa pod husks and green tea contain high polyphenols which are useful for their antibacterial, anti-inflammatory, and antioxidant properties.

The extracts of cocoa pod husk and green tea combined with calcium hydroxide are expected to increase the effectiveness of calcium hydroxide as a pulp capping material.

Objectives to prove the extracts of cocoa pod husk and green tea's effects on the SMAD3 and FGF2 expressions in mice with perforated dental pulps.

A total of 54 rats were used and divided into three groups: given calcium hydroxide treatment with distilled water, calcium hydroxide with the extract of cocoa pod husk, and calcium hydroxide with the extract of green tea. Then, cavities were then restored. The experimental animals from each treatment group were killed on days 3, 7, and 14 to observe their SMAD3 and FGF2 expressions.

Data analysis with the Tukey HSD test for SMAD3 and FGF2 expressions on the two test groups suggested no significant difference. The extracts of cocoa pod husk and green tea have the same ability to increase the SMAD3 and FGF2 expression in exposed dental pulp.

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Introduction

Dental pulp is a connective tissue consisting of nerves, blood vessels, basic substances, interstitial fluid, odontoblasts, fibroblasts, and other cell components.[1] Pulp tissue can be affected by various stimuli, such as deep caries and trauma affected by accidental preparation techniques used during tooth restoration.[2] Perforation in the pulp can cause a homeostatic imbalance of the pulp. The pulp responds histologically, and it is characterized by inflammation, synthesis of new collagen tissue, and formation of dentine bridges that end with the final formation of reparative dentin.[3]

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Fibroblast Growth Factor-2 (FGF2) is a growth factor with a crucial role in the regeneration of the dentin-pulp complex. In regulating tooth morphogenesis, FGF2 plays a role by controlling cell differentiation and proliferation. As a strong angiogenic factor, FGF2, along with PDGF and VEGF, stimulates the new blood vessels' formation in the dental pulp. While FGF2 also induces the dental pulp cells' migration and stimulates the tooth pulp cells' proliferation without differentiation, the synergy between FGF2 and TGFβ1 induces the differentiation in dental pulp cells into odontoblast-like cells and increases the effect of TGFβ1 on odontoblast differentiation. FGF2 stimulates the formation of dentin-like particles on exposed pulp.[4] SMAD is a protein involved in TGFβ1 signaling. SMAD3 is one of the SMAD groups involved in TGFβ1 signaling.[5] Functional signaling from SMAD3 is required for FGF2 mRNA induced TGF2 and protein expression in fibroblasts.[6]

Pulp capping refers to an exposed vital pulp's treatment caused by mechanical or chemical factors. It is done by placing dental materials over an open area to facilitate barrier formation and maintain pulp vitality.[7] The vitality of the dentin-pulp complex needs to be maintained because it is fundamental to the functional life of the teeth.[8] The success of the pulp closure procedure is highly determined by the covering material's capacity to completely cover the tubules, not cause irritation, protect the pulp from bacterial, chemical and mechanical irritants, and induce odontoblast and fibroblast cells to establish perfect dentin and its bridges.[9]

Calcium hydroxide [$Ca(OH)_2$] is recommended as a direct pulp capping material because it has beneficial properties which can stimulate mineralization (recovery process) and inhibit bacterial growth.[10] However, calcium hydroxide also has some drawbacks such as dissolving clinically within 1-2 years; being susceptible to dissolution by acids and tissue fluids; and causing problems during acid etching resin restoration. As many as 89% of the dentin bridges formed have tunnel defects at high risk of causing micro-leakage, which leads to re-infection of bacteria, continued pulp inflammation, and necrosis.[11]

Cocoa is a natural ingredient that contains polyphenols consisting of condensed tannins that have antioxidant, anti-inflammatory, and antimicrobial properties.[12,13,14] Its effectiveness as an antibacterial has been proven against the *Enterococcus faecalis.*[15] Cocoa pod husks have a much higher total phenolic content and a higher antioxidant effect than other parts of cocoa.[16] Proanthocyanidin is the most prevalent polyphenol in cocoa, wherein it makes up 58%-65% of the total polyphenols in cocoa pods.[17,18] This substance acts as an anti-inflammatory agent by inhibiting the release of arachidonic acid and as lysosomal enzymes by blocking the cyclooxygenase and lipoxygenase pathways in the process of inflammation. The suppression of prostaglandins and leukotrienes as inflammatory mediators causes the migration of inflammatory cells to the wound area to be reduced, allowing it to suppress the inflammatory response.[19] Moreover, proanthocyanidin works as an antioxidant by interfering with one of Fenton's reactions, thereby inhibiting oxidation.[20] Previous research has shown that the application of proanthocyanidin in direct contact with the pulp can increase cell metabolism and collagen synthesis, as well as act as a biostimulator and protect dental pulp cells.[21] Other research has proven the effectiveness of cocoa pod husks on reparative dentin formation.[15]

Green tea is a source of antioxidants rich in polyphenols, which have anti-inflammatory, antioxidant, antibacterial, antiviral and anti-fungal properties.[22] EGCG is the main polyphenol of green tea, accounting for about 59% of total catechins.[23] Several studies have shown that EGCG polyphenols are active flavonoid compounds with important role in cutting the inflammation's period thanks to its high antioxidant. They do this by reducing levels of nitric oxide and slowing down the creation of inducible nitric oxide synthase (iNOS) to reduce inflammatory cells in the wound area and shorten the inflammatory reaction. This then allows the proliferation phase to begin; it means the tissue repair and healing process will occur quicker.[24] The ability of EGCG to scavenge free radicals by providing stable phenol radicals is due to the presence of galloyl groups in the B and D rings that can simultaneously capture free OH and O_2 radicals.[25] Macrophages, one of the cells responsible for tissue repairing, begin the proliferation phase by releasing factors that stimulate repair and healing growths. It was found in the previous studies that the combination between EGCG and hydrogel dosage form are biocompatible and are beneficial to induce tissue regeneration and the release of FGF-2.[24] Other research has found the effectiveness of green tea on reparative dentin formation.[26]

Objectives to prove the effect of cocoa pod husk and green tea extract on the SMAD3 and FGF2 expressions in mice with perforated dental pulps.

Materials and methods

Research Samples

The total sample size of this study was 54 males *Rattus novergicus* aged 12-16 weeks with a body weight of 250-300 grams. The samples were divided randomly into three groups consisting of 18 male rats each. The concentrations of the extracts of cocoa pod husk and green tea used in this study were 3.125% and 0.8%, respectively.

Research Methods

The positive control group (group 1) was given calcium hydroxide with distilled water at 1:1 ratio; the next group was given calcium hydroxide with the extract of cocoa pod husk at 1:1 ratio (group 2), and the last group was given calcium hydroxide with the extract of green tea at 1:1 ratio (group 3). The material was placed on the pulps' surface, and the cavity was restored. The mice were returned to the cages after being tagged.

To observe the activation of SMAD3 and FGF2, 18 mice were killed on the third day. The other 18 mice were killed on the seventh day, and the other 18 mice were killed on the 14th day. On each day, six mice were placed for each treatment group. The jaw bones in the interdental area of the right maxillary first molar were taken to carry out the histopathology process. Histological preparations were obtained with a thickness of ± 4µm parallel to the long axis of the tooth. Each specimen produced two slides to be observed.

Immunohistochemical (IHC) observations on SMAD3 and FGF2 activation were performed using primary antibodies (monoclonal mouse) (Santa Cruzz B) to focus on protein and antibody binding. Using the chromogen substrate, the enzyme was reacted and then observed with an Olympus BX51 light microscope with a magnification of 1,000 times and a calculation of 20x the field of view.

Statistical methods

The data obtained from this study were presented in the form of mean and standard deviation. To determine the distribution and homogeneity of data, the Kolmogorov-Smirnov Lilliefors test was used, and the next was the homogeneity Levene test. The ANOVA test was used to see if treatment group experienced significant differences, and the Tukey HSD test was used to assess the differences with a significance level of 0.05.

Results

Immunohistochemical (IHC) detection of SMAD3 on days 3, 7, and 14 can be seen on Figures 1-3. The arrows show activated SMAD3 on fibroblast cells in all groups: A. Group 1; B. Group 2; C. Group 3. The average SMAD3 expression in each treatment group is shown in Graph 1. The highest mean value of SMAD3 expression in each group was on day 14. The average was 8.50 in Group 1; 14 in Group 2; and 15 in Group 3.

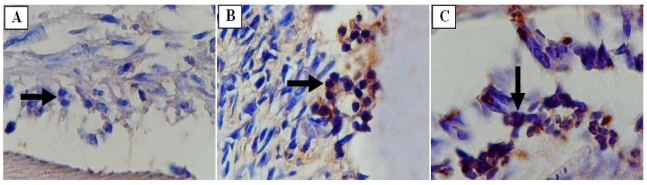

Figure 1. IHC detection of SMAD3 expression on day 3. The arrows show activated SMAD3 on fibroblast cells. A. Group 1; B. Group 2; C. Group 3.

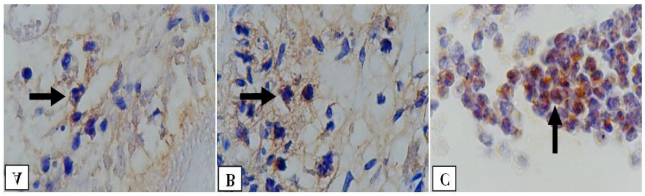

Figure 2. IHC detection of SMAD3 on day 7. The arrows show activated SMAD3 on fibroblast cells. A. Group 1; B. Group 2; C. Group 3.

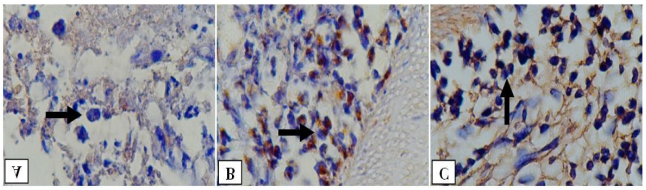

Figure 3. IHC detection of SMAD3 on day 14. The arrows show activated SMAD3 on fibroblast cells. A. Group 1; B. Group 2; C. Group 3.

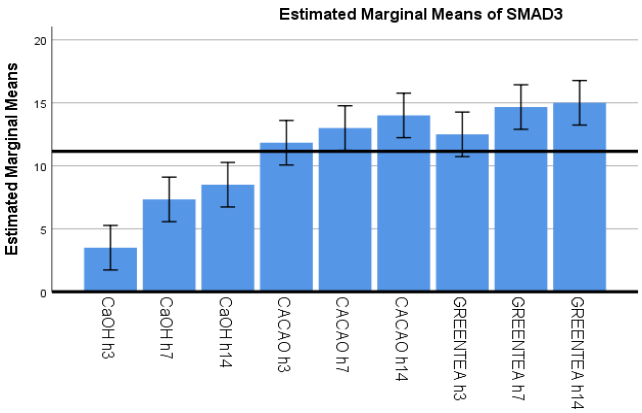

Figure 4. The mean value of SMAD3 activation on days 3, 7, and 14.

IHC detection of FGF2 on day 3, 7, and 14 can be seen on Figure 4-6. The arrows show activated FGF2 on fibroblast cells in each group. A. Group 1; B. Group 2; C. Group 3. The average

FGF2 expression in each treatment group is shown in Graph 2. The highest mean value of FGF2 expression in each group was on day 14. The average was 9 in Group 1; 14.83 in Group 2; and 15 in Group 3.

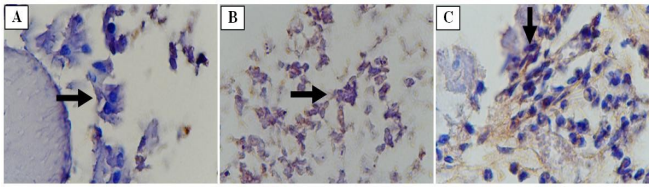

Figure 5. IHC detection of FGF2 on day 3. The arrows show activated FGF2 on fibroblast cells. A. Group 1; B. Group 2; C. Group 3.

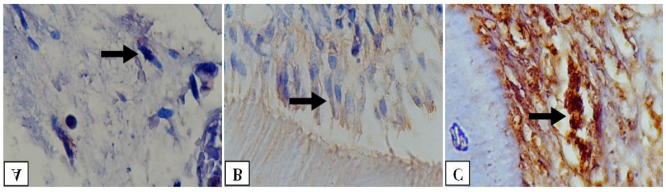

Figure 6. IHC detection of FGF2 on day 7. The arrows show activated FGF2 on fibroblast cells. A. Group 1; B. Group 2; C. Group 3.

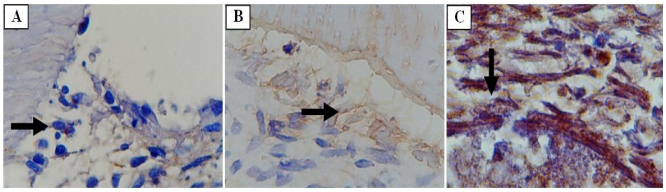

Figure 7. IHC detection of FGF2 on day 14. The arrows show activated FGF2 on fibroblast cells. A. Group 1; B. Group 2; C. Group 3.

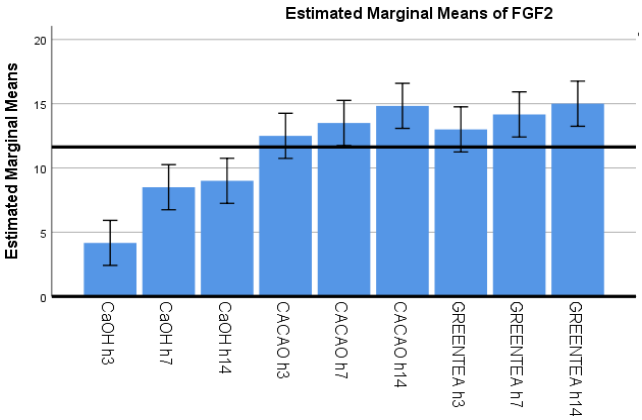

Figure 8. The mean value of FGF2 activation on days 3, 7, and 14

Based on the normality and homogeneity tests on SMAD3 and FGF2 activation, data were normally distributed and homogeneous. Furthermore, the ANOVA test determined the effect of SMAD3 and FGF2 activation on the treatment groups.

Sum of Squares		df	Mean Square	F	Sig.
Between Groups	726.815	8	90.852	19.655	.000

Table 1. ANOVA test for Smad3 expression.

Sum of Squares		df	Mean Square	F	Sig.
Between Groups	645.148	8	80.644	17.688	.000

Table 2. ANOVA test for FGF2 expression.

In this study, the ANOVA result on the activation of SMAD3 (Table 1 and Table 2) revealed that the F value between groups had a significance value of 0.000 ($p < 0.05$). It means that the combination between the treatment groups on SMAD3 and FGF2 expressions suggested a significant effect.

Based on the SMAD3 activation, significant differences were found between Group 1 and Group 2 on days 3, 7, and 14 ($p < 0.05$). However, there was no significant difference between Group 1 on day 14 and Groups 2 and 3 on day 3 ($p > 0.05$). Insignificant differences were also found between Group 2 and Group 3 on days 3, 7, and 14 ($p > 0.05$) (Table 3).

Groups	$Ca(OH)_2$ + aquadest D3	$Ca(OH)_2$ + aquadest D7	$Ca(OH)_2$ + aquadest D14	$Ca(OH)_2$ + Cocoa D3	$Ca(OH)_2$ + Cocoa D7	$Ca(OH)_2$ + Cocoa D14	$Ca(OH)_2$ + Green tea D3	$Ca(OH)_2$ + Green tea D7	$Ca(OH)_2$ + Green tea D14
$Ca(OH)_2$ + aquadest D3	-								
$Ca(OH)_2$ + aquadest D7	0.075	-							
$Ca(OH)_2$ + aquadest D14	0.006*	0.989	-						
$Ca(OH)_2$ + Cocoa D3	0.000*	0.019*	0.182	-					
$Ca(OH)_2$ + Cocoa D7	0.000*	0.001*	0.019*	0.989	-				
$Ca(OH)_2$ + Cocoa D14	0.000*	0.000*	0.002*	0.716	0.996	-			
$Ca(OH)_2$ + Green tea D3	0.000*	0.004*	0.054	1.000	1.000	0.950	-		
$Ca(OH)_2$ + Green tea D7	0.000*	0.000*	0.000*	0.374	0.913	1.000	0.716	-	
$Ca(OH)_2$ + Green tea D14	0.000*	0.000*	0.000*	0.236	0.794	0.996	0.542	1.000	-

Table 3. Tukey HSD Test for SMAD3 expression on days 3, 7, and 14. The different marks *are statistically significant ($p < 0.05$). D: day.

Significant differences were found in the activation of FGF2 for Group 1 on day 3 compared to Groups 2 and 3 on days 3, 7, and 14 ($p < 0.05$). Significant differences were also

found between Group 1 on days 7 and 14 compared to Groups 2 and 3 on days 7 and 14 ($p < 0.05$). However, no significant differences were found between Group 1 on days 7 and 14 compared to Groups 2 and 3 on day 3 ($p > 0.05$). Insignificant differences were also found between Group 2 and Group 3 on days 3, 7, and 14 ($p > 0.05$) (Table 4).

Groups	$Ca(OH)_2$ + aquadest D3	$Ca(OH)_2$ + aquadest D7	$Ca(OH)_2$ + aquadest D14	$Ca(OH)_2$ + Cocoa D3	$Ca(OH)_2$ + Cocoa D7	$Ca(OH)_2$ + Cocoa D14	$Ca(OH)_2$ + Green tea D3	$Ca(OH)_2$ + Green tea D7	$Ca(OH)_2$ + Green tea D14
$Ca(OH)_2$ + aquadest D3	-								
$Ca(OH)_2$ + aquadest D7	0.026*	-							
$Ca(OH)_2$ + aquadest D14	0.008*	1.000	-						
$Ca(OH)_2$ + Cocoa D3	0.000*	0.052	0.132	-					
$Ca(OH)_2$ + Cocoa D7	0.000*	0.002*	0.008*	0.974	-				
$Ca(OH)_2$ + Cocoa D14	0.000*	0.000*	0.001*	0.622	0.996	-			
$Ca(OH)_2$ + Green tea D3	0.000*	0.026*	0.072	1.000	0.996	0.788	-		
$Ca(OH)_2$ + Green tea D7	0.000*	0.001*	0.004*	0.909	1.000	1.000	0.974	-	
$Ca(OH)_2$ + Green tea D14	0.000*	0.000*	0.000*	0.533	0.989	1.000	0.708	0.999	-

Table 4. Tukey HSD test for FGF2 expression on days 3, 7, and 14. The different marks * are statistically significant ($p < 0.05$) D: day

Discussion

The result on the activation of SMAD3 and FGF2 on days 3, 7, and 14 showed that the effect of the combination of $Ca(OH)_2$ and distilled water were found to have the lowest mean value in comparison to the other two groups. The highest mean value was shown in the combination of $Ca(OH)_2$ and the extract of green tea, followed by the combination of $Ca(OH)_2$ and the extract of cocoa pod husk with an insignificant difference.

This condition indicates that the extracts of cocoa pod husk and green tea used as a mixing agent for calcium hydroxide increases SMAD3 and FGF2 activation as the average of SMAD3 and FGF2 activation was higher on each day in both groups compared to the combination of $Ca(OH)_2$ with distilled water. The results of this study also showed the increase in SMAD3 activation, which went along with the increase of FGF2 activation on days 3, 7, and 14. These findings are in line with research conducted by Strand et al. spotting that SMAD3 could regulate the expression of FGF2 induced by TGFβ1.[8]

The increase in FGF2 expression on day 14 obtained in this study is not in line with the study by Sagomonyants and Mina, showing the stage of the specific effect of FGF2 on tooth pulp cell differentiation from day 3 to day 21.[27] In this study, the expression of FGF2 peaked on the seventh day, it then decreased on the 14th day and was still expressed in a fixed amount until the 21st day.

However, this study found that the increase in FGF2 expression continued to occur until day 14 in all treatment groups most likely due to the different types of cells studied. Sagomonyants and Mina observed the expression of FGF2 in odontoblast cells; meanwhile, in this study, the expression of FGF2 was seen in fibroblast cell; whilst the pulp was exposed, the proliferation of fibroblast cells still increased until day 21.[27] Besides, the use of calcium hydroxide as the base material for pulp capping caused an increase in extracellular Ca^{2+} levels, which triggered an increase in FGF2 expression. According to Kanaya et al., the use of calcium hydroxide and other calcium-based materials for pulp capping treatments, both direct and indirect, will likely increase extracellular Ca^{2+} levels, which can likely incline in the expression of the FGF2 gene in hDP (human dental papilla) and mDP (mouse dental papilla) through PKA and ERK 1/2.[28]

The highest average value of SMAD3 and FGF2 activation on the group combination of $Ca(OH)_2$ and the extract of green tea was most likely due to the high content of polyphenols in green tea extract, which was dominated by EGCG.[21] Epigallocatechin-3-gallate has anti-inflammatory effects that can inhibit the translation of NF-kB into the nucleus. According to previous studies, epigallocatechin-3- gallate (EGCG) will inhibit the IKK/NF-kB signal transduction pathway because it inhibits IKK phosphorylation. This results in inhibited IkB inhibition, decreased NF-kB activity, and inhibited TNF-a expression that limit the number of migrating inflammatory cells to the wound.[29] This condition results in a shorter inflammatory reaction.

Besides, EGCG also has antioxidant properties that can inhibit iNOS production and reduce NO levels,[24] as well as inhibit TNF-induced ROS and cell death by supporting complex dissociation. The released nuclear

factor E2-related factor2 (Nrf2) will translocate to the nucleus that will activate the genes transcription, along with other transcription factors, which contain the antioxidant response element (ARE) in the promoter region. It will then produce an inhibitory effect on NF-κB activity. In contrast, the Kelch-like ECH-associated protein 1 (Keap1) is intersected from the Nrf2-Keap1 complex and interacts directly with IKKβ and suppresses NF-κB function. Moreover, Nrf2, which translocate to the nucleus and binds to ARE elements, will encourage various enzymes or antioxidant proteins activations that can decrease oxidative stress.[30,31]

Hydroxyl groups or -OH groups originate the presence of easily-oxidized antioxidant properties of EGCG in ring B. They likely open oxygen atoms, raise reactivity to biological polymeric and heavy metal bonds, catalyze electron transport, and seize free radicals including nitric oxide (NO). Produced by the enzyme nitric oxide synthase (NOS), NO is a free radical formed by the use of burs which resulted in friction and heat when preparations were made in deep cavities. As a result, the blood vessels vasoconstricted and the capillary permeability decreased. It then corresponded to the decrease in neutrophil cell migration, allowing a quicker phase of the acute inflammation in the injury area. As the number of macrophages increases, the macrophage activity raises as a second defense by carrying out the phagocytosis process, cleaning debris tissue, then releasing and activating growth factors such as PDGF, TGFβ and FGF to stimulate the proliferation and migration of fibroblasts cells.[32]

There was an insignificant difference in Smad3 and FGF2 expression between the combination group of $Ca(OH)_2$ with the extract of cocoa pod husk and the combination of $Ca(OH)_2$ and with the extract of green tea on days 3, 7, and 14. This can be attributed to the high polyphenol content dominated by proanthocyanidin in the cocoa pod husk extract, which has anti-inflammatory and antioxidant effects.[33] Besides, proanthocyanidin works as an antioxidant by interfering with one of Fenton's reactions, thereby inhibiting oxidation. Cocoa pod husks also have antioxidant properties because they contain the alkaloid purine class, namely theobromine. Similar to proanthocyanidin, theobromine works by intervening in one of the Fenton reactions to inhibit the oxidation reaction.[20,34]

Having cocoa flavonoids as antioxidants is related to their structure. The orthodihydroxy 3', 4' (catechol) structure on the B ring provides stability to the penoxyl radicals through hydrogen bonding or electron delocalization. The catechol structure in ring B increases the inhibition of fat peroxide and plays a role in capturing peroxyl, superoxide, and peroxynitrite radicals. This structureless flavonoid compound has low antioxidant activity. The C2, C3 double bonds and the conjugation with the 4-oxo group on the C ring are responsible for radical stabilization through electron delocalization in all three rings. The presence of hydroxyl Groups 3 and 5 could increase antioxidant activity. Compounds that do not have an orthodihydroxy structure in ring B but have a catechol structure in ring A show greater antioxidant activity.

Conclusions

Cocoa pod husk and green tea extract have the same ability to increase the SMAD3 and FGF2 expressions in exposed dental pulp.

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Declaration of Interest

The authors declare that there are no conflicts of interest.

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