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Quantitative assessment of enamel color alterations induced by herbal bleaching agents using spectrophotometry

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Abstract:
The demand for safer natural tooth-whitening agents has increased, necessitating evaluation of their bleaching efficacy. Therefore, it is of interest to compare the bleaching efficacy of strawberry fruit extract, guava leaf extract and 70% dark chocolate with 3% hydrogen peroxide on artificially stained human enamel using spectrophotometric analysis. Forty extracted molar teeth were allocated into four groups (n=10) and color changes were assessed on Days 1 and 7 using a CIE Lab spectrophotometer. Significant differences were observed among the groups ($p<0.001$), with 3% hydrogen peroxide showing the highest bleaching efficacy, followed by strawberry fruit extract, guava leaf extract and 70% dark chocolate. Thus, strawberry fruit extract was the most effective natural bleaching agent, while further clinical studies are needed to confirm these findings.

Keywords: Guava leaf extract, hydrogen peroxide, spectrophotometry, strawberry fruit extract, tooth bleaching, tooth discoloration

Background:
Dental discoloration is a common esthetic concern and may be intrinsic or extrinsic, with tooth color influencing appearance and self-confidence **[1]**. Intrinsic discoloration is associated with factors such as aging, trauma, medications, developmental defects and systemic conditions, whereas extrinsic staining commonly results from dietary products, tobacco, tea, coffee and paan **[2]**. Tooth bleaching is a conservative approach for improving tooth color, with hydrogen peroxide remaining the standard agent because of its strong oxidative action **[3]**. However, peroxide-based bleaching may cause tooth sensitivity, enamel surface alterations, increased roughness, porosity and soft-tissue irritation **[4]**. These concerns have increased interest in plant-based whitening agents containing organic acids, flavonoids, polyphenols, tannins and antioxidants **[5]**. Strawberry extract may promote stain removal through its malic acid content, while guava leaves contain flavonoids and polyphenols that may improve enamel cleanliness and optical appearance **[5]**. Conversely, the whitening effect of dark chocolate, despite its polyphenol and pigment content, remains poorly investigated **[6].** Therefore, it is of interest to report the comparative bleaching efficacy of strawberry fruit extract, guava leaf extract, 70% dark chocolate and 3% hydrogen peroxide on artificially stained human enamel using spectrophotometric analysis.

Materials and Methods:
Study design:
This *in vitro* experimental study was conducted in the Department of Conservative Dentistry and Endodontics to evaluate and compare enamel color recovery in artificially stained human teeth following treatment with herbal extracts and conventional hydrogen peroxide using spectrophotometric analysis. The study was designed in accordance with the recommendations of the Checklist for Reporting *in vitro* studies (CRIS) to ensure transparency, methodological rigor and reproducibility. The study protocol was reviewed and approved by the Institutional Ethics Committee prior to commencement of the investigation (Approval No. CKS/IEC-ENDO/2026/30). Although the study involved extracted human teeth and did not include direct patient intervention, ethical approval was obtained in accordance with institutional guidelines governing the use of human biological specimens for research. All teeth were collected after obtaining written informed consent from the donors permitting the use of the extracted teeth for research purposes.

Sample size calculation:
The sample size was estimated using G*Power software (version 3.1.9.7; Heinrich Heine University, Düsseldorf, Germany) for one-way analysis of variance (ANOVA) involving four independent groups. Enamel color change (ΔE) was considered the primary outcome variable. Assuming a significance level (α) of 0.05, a statistical power of 80% and a large effect size ($f = 0.60$), the minimum required sample size was calculated to be 36 specimens. To compensate for possible specimen loss during sample preparation, staining procedures or spectrophotometric analysis, the sample size was increased to 40 specimens, with 10 teeth allocated to each experimental group.

Sample collection and preparation:
Forty freshly extracted permanent human molars extracted for periodontal or orthodontic reasons were collected from the Department of Oral and Maxillofacial Surgery. Teeth presenting intact enamel surfaces without caries, restorations, developmental defects, fluorosis, cracks, fractures or excessive wear were included in the study. Teeth exhibiting intrinsic discoloration, hypoplastic defects, restorations, or previous bleaching treatment were excluded. Following extraction, all soft tissue remnants and calculus deposits were removed using an ultrasonic scaler. The specimens were disinfected by immersion

in 0.1% thymol solution for 24 hours and subsequently stored in distilled water at room temperature until use. The storage medium was replaced weekly to minimize microbial contamination and prevent dehydration of the dental tissues.

Preparation of artificial staining solution:
Artificial staining solutions were prepared using three commonly consumed chromogenic substances known to produce extrinsic dental staining, namely herbal tea, coffee and paan (betel leaf). Four grams of herbal tea powder, instant coffee powder and paan extract were each dissolved separately in 100 mL of boiling distilled water and allowed too steep for five minutes. The solutions were filtered through Whatman No. 1 filter paper to remove suspended particles and cooled to room temperature. Equal volumes of the three prepared solutions were then combined to obtain a standardized staining solution. Fresh staining solution as prepared on the day of the experiment to ensure consistency in chromogen concentration.

Artificial staining procedure:
Each tooth specimen was completely immersed in the freshly prepared staining solution and incubated at 37°C for 24 hours to simulate standardized extrinsic discoloration. Following the staining procedure, all specimens were thoroughly rinsed under distilled water to remove loosely adherent pigments and stored in 100% humidity at 37°C until further evaluation. Standardization of the staining protocol ensured uniform discoloration of all specimens before treatment allocation.

Randomization and allocation:
Each specimen was assigned a unique identification number and randomly allocated into four experimental groups using a computer-generated randomization sequence. Allocation was performed by an independent investigator who was not involved in color assessment to minimize allocation bias. The examiner responsible for spectrophotometric measurements remained blinded to the treatment allocation throughout the study. Each experimental group consisted of ten specimens, which were further divided into two equal subgroups based on the treatment duration of one day and seven consecutive days.

Preparation of experimental agents:
Fresh strawberry extract was prepared by washing ripe strawberries thoroughly with distilled water, removing the calyx and homogenizing the fruits using a sterile laboratory blender. The homogenate was filtered sequentially through sterile muslin cloth and Whatman No. 1 filter paper to obtain a uniform extract, which was prepared fresh each day of the experiment. Fresh guava leaves were thoroughly washed, shade dried and pulverized into a fine powder. Ten grams of the powdered leaves were mixed with 100 mL of distilled water and heated at 60°C for 30 minutes. After cooling to room temperature, the mixture was filtered through Whatman No. 1 filter paper and the filtrate served as the aqueous guava leaf extract. Fresh extract was prepared daily to preserve its phytochemical activity. Commercially available 70% dark chocolate was melted in a thermostatically controlled water bath maintained at approximately 40°C until a uniform consistency was achieved. Freshly melted chocolate was prepared immediately before each treatment cycle. Commercially available 3% hydrogen peroxide solution served as the conventional bleaching agent and was used according to the manufacturer's recommendations.

Treatment protocol:
The stained specimens were allocated into four experimental groups comprising hydrogen peroxide, strawberry extract, guava leaf extract and dark chocolate. Each group was further subdivided into two subgroups according to treatment duration. In subgroup a, specimens were immersed in 20 mL of the respective treatment solution for eight hours daily for one day, whereas specimens in subgroup B underwent immersion for eight hours daily for seven consecutive days. Fresh treatment solutions were prepared before each immersion cycle to maintain consistency in the concentration of the active ingredients. During the remaining sixteen hours of each day, all specimens were stored individually in distilled water maintained at 37°C to simulate oral hydration and prevent dehydration of the enamel surface.

Post-treatment color assessment:
Following completion of the assigned treatment regimen, all specimens were rinsed thoroughly with distilled water and gently air dried. Final color measurements were obtained using the same spectrophotometer (Konica Minolta CM5 2021, Tokyo, Japan), measurement site and calibration protocol employed during baseline evaluation. Three consecutive readings were recorded for each specimen and the mean values were used for analysis. The overall color difference (ΔE) was calculated from the CIE Lab* coordinates using the standard formula:

$$(\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2})$$

Where (L_1), (a_1) and (b_1) represent the baseline values and (L_2), (a_2) and (b_2) represent the post-treatment values. Outcome Measure The primary outcome measure was enamel color recovery, expressed as the change in ΔE values between day 1 and day 7 spectrophotometric assessments. Greater reductions in ΔE values were interpreted as improved recovery of enamel color following treatment.

Statistical analysis:
The data were analyzed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). Intergroup comparisons at each evaluation period (Day 1 and Day 7) were performed using One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc test for pairwise group comparisons. Intragroup comparisons between Day 1 and Day 7 were performed using repeated-measures ANOVA. A p value < 0.05 was considered statistically significant.

Results:

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One-way ANOVA showed a statistically significant difference in ΔE values among the four study groups on Day 1 (F (3, 36)=486.64, p = 0.001). The highest mean ΔE value was observed in the 3% H_2O_2 group, followed by strawberry extract, guava leaf extract and 70% dark chocolate, indicating that 3% H_2O_2 produced the greatest immediate bleaching effect **(Table 1)**. One-way ANOVA revealed a statistically significant difference in ΔE values among the four study groups on Day 7 (F (3, 36) = 438.72, p=0.001). The highest mean ΔE value was observed in the 3% H_2O_2 group, followed by strawberry extract, guava leaf extract and 70% dark chocolate. These findings indicate that 3% H_2O_2 showed the greatest bleaching efficacy on Day 7, whereas 70% dark chocolate exhibited the least color change **(Table 2).** Repeated-measures ANOVA demonstrated a statistically significant increase in ΔE values from Day 1 to Day 7 in all study groups (p < 0.05). Significant increases were observed in the 3% H_2O_2 (p = 0.001), strawberry extract (p = 0.001), guava leaf extract (p = 0.002) and 70% dark chocolate (p = 0.003) groups. These findings indicate progressive bleaching over time in all groups, with 3% H_2O_2 exhibiting the greatest color change **(Table 3).** Tukey's post hoc analysis demonstrated statistically significant pairwise differences among all four treatment groups on Day 1 (all p = 0.001). The 3% H_2O_2 group exhibited significantly greater ΔE values than the strawberry extract (mean difference = 1.03), guava leaf extract (mean difference = 3.06) and 70% dark chocolate groups (mean difference = 5.77). Furthermore, the strawberry extract group showed significantly higher ΔE values than the guava leaf extract (mean difference = 2.03) and 70% dark chocolate groups (mean difference = 4.74). The guava leaf extract group also demonstrated significantly greater bleaching efficacy than the 70% dark chocolate group (mean difference = 2.71). These findings indicate that all bleaching agents differed significantly from one another on Day 1, with 3% H_2O_2 producing the greatest color change, followed by strawberry extract, guava leaf extract and 70% dark chocolate **(Table 4).** Tukey's post hoc analysis revealed statistically significant pairwise differences in ΔE values among all study groups on Day 7 (all p = 0.001). The 3% H_2O_2 group demonstrated significantly greater color change than the strawberry extract, guava leaf extract and 70% dark chocolate groups. Similarly, strawberry extract produced significantly higher ΔE values than guava leaf extract and 70% dark chocolate, while guava leaf extract was significantly superior to 70% dark chocolate. These results confirm that the bleaching efficacy on Day 7 was highest for 3% H_2O_2, followed by strawberry extract, guava leaf extract and 70% dark chocolate, with statistically significant differences between all pairwise comparisons (**Table 5).**

Table 1: Intergroup comparison for day 1

Groups	N	Mean ± SD	df	F value	P value
3% H2O2	10	5.91 ± 0.31	3	486.64	
Strawberry extract	10	4.88 ± 0.09			0.001*
Guava Leaf extract	10	2.85 ± 0.40			
70% Dark chocolate	10	0.14 ± 0.06			

One-way ANOVA; p < 0.05 considered statistically significant

Table 2: Intergroup comparison for day 7

Groups	N	Mean ± SD	df	F value	P value
3% H2O2	10	6.86 ± 0.22	3	438.72	
Strawberry extract	10	6.21 ± 0.21			0.001*
Guava Leaf extract	10	4.16 ± 0.08			
70% Dark chocolate	10	0.74 ± 0.18			

One-way ANOVA; p <0.05 considered statistically significant

Table 3: Comparison between 2 different timelines

Groups	N	Day 1 Mean ± SD	Day 7 Mean ± SD	Mean Difference	P value
3% H2O2	10	5.91 ± 0.31	6.86 ± 0.22	−0.94	0.001*
Strawberry extract	10	4.88 ± 0.09	6.21 ± 0.21	−1.33	0.001*
Guava Leaf extract	10	2.85 ± 0.40	4.16 ± 0.08	−1.31	0.002*
70% Dark chocolate	10	0.14 ± 0.06	0.74 ± 0.18	−0.59	0.003*

Repeated Measure ANOVA; p<0.05 considered statistically significant

Table 4: Tukey's Post Hoc test at Day 1

Group	Group	Mean Difference	Std Error	Sig.
3% H2O2	Strawberry extract	1.030	0.528	0.001*
	Guava Leaf extract	3.060	0.528	0.001*
	70% Dark chocolate	5.770	0.528	0.001*
Strawberry extract	Guava Leaf extract	2.030	0.528	0.001*
	70% Dark chocolate	4.740	0.528	0.001*
Guava Leaf Extract	70% Dark chocolate	2.710	0.528	0.001*

p<0.05 considered statistically significant

Table 5: Tukey's Post Hoc test at Day 7

Group	Group	Mean Difference	Std Error	Sig.
3% H2O2	Strawberry extract	0.65	0.115	0.001*
	Guava Leaf extract	2.69	0.115	0.001*
	70% Dark chocolate	6.12	0.115	0.001*
Strawberry extract	Guava Leaf extract	2.04	0.115	0.001*
	70% Dark chocolate	5.47	0.115	0.001*
Guava Leaf Extract	70% Dark chocolate	3.42	0.115	0.001*

p<0.05 considered statistically significant

Discussion:

Tooth discoloration is a common esthetic concern caused by intrinsic and extrinsic factors, including aging, trauma, dietary habits, tobacco exposure and chromogenic beverages **[7].** Tooth bleaching is a conservative and widely used approach for improving tooth color, with hydrogen peroxide and carbamide peroxide being the principal bleaching agents used in clinical practice **[8].** Their whitening effect results primarily from oxidation of chromogenic compounds within dental tissues, producing lighter tooth color. However, bleaching may cause adverse effects, particularly tooth sensitivity, with higher peroxide concentrations associated with increased sensitivity without providing substantially greater clinical color change **[9].** Recent evidence indicates that reducing the exposure time of at-home bleaching gels can decrease tooth sensitivity, although prolonged exposure may produce greater color change **[10].** Bleaching agents may also produce changes in enamel morphology and permeability, including increased surface roughness and alterations in mineral and organic components, particularly depending on concentration and treatment conditions **[11].** These limitations have encouraged interest in alternative and minimally abrasive whitening approaches. Whitening dentifrices, for example, have showed significant reductions in the area and intensity of extrinsic stains compared with conventional dentifrices **[12].** Natural products containing

organic acids, flavonoids, polyphenols, tannins and other bioactive compounds have consequently attracted attention as potential alternatives for stain removal. However, evidence regarding the effectiveness and safety of specific plant- and food-derived whitening agents remains limited compared with conventional peroxide-based systems **[13].** Therefore, controlled laboratory investigations using objective spectrophotometric color assessment are warranted to determine whether naturally derived materials can produce clinically relevant whitening effects. The present study consequently evaluates the whitening potential of selected natural products, including strawberry and guava leaf preparations and compares their effects with conventional bleaching approaches.

Conclusion:

3% hydrogen peroxide showed the highest bleaching efficacy, significantly outperforming all tested natural agents. Among the natural alternatives, strawberry fruit extract showed the greatest whitening potential, followed by guava leaf extract, while 70% dark chocolate exhibited negligible effects. Further studies are warranted to establish the clinical effectiveness, safety, and potential of natural bleaching agents as alternatives or adjuncts to conventional treatments.

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