Original Article

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Dentistry Section

Effectiveness of Two Phytochemicals, P-Coumaric Acid and Quercetin in Reducing the Melanin Content of Pigmented Gingival Tissue: An Ex-vivo Study

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ABSTRACT

Introduction: Pigmented gingiva is an aesthetic concern for many individuals. Various treatment modalities have been developed to address this condition, but each carries certain disadvantages. Recently, the use of herbal products has been considered as a treatment modality, as they are safe and readily available. P-Coumaric Acid (p-CA) and Quercetin are two plant-based chemicals that have shown depigmenting effects on the skin. Given the similar cellular structure of skin and gingiva, the present study aimed to test their depigmenting effects on the gingiva.

Aim: To evaluate the effectiveness of two phytochemicals, p-CA (3 µM) and Quercetin (20 µM), in reducing the melanin content of pigmented gingival tissue.

Materials and Methods: The study was an ex-vivo study conducted in the Department of Periodontology at Bharati Vidyapeeth Deemed to be University Dental College and Hospital, Pune, Maharashtra, India, from January 2024 to April 2024. A total of 10 patients with a Dummet Oral Pigmentation Index (DOPI) score of two were selected for the study. A surgical depigmentation procedure was performed and the excised tissue was divided into three parts. The first part of the excised tissue served as a control group (Group A), while the second and third parts were treated with p-CA (Group B) and Quercetin (Group C), respectively. Histological analysis was conducted using Haematoxylin and Eosin (H&E) staining and immunohistochemical analysis was performed using the Human Melanoma Black 45 (HMB-45) antibody test. The reduction in melanin content was recorded in all three groups and the results obtained were compared using a t-test. Statistical Package for the Social Sciences (SPSS) version 20.0 was used for statistical analysis, with a p-value of ≤0.05 considered statistically significant.

Results: Both p-CA and Quercetin demonstrated a reduction in the level of pigmentation. Comparative analysis indicated that p-CA had a stronger effect than Quercetin.

Conclusion: The p-CA and Quercetin can be considered potential depigmenting agents.

Keywords: Flavonoids, Gingiva, Hyperpigmentation, Immunohistochemistry, Melanocytes

INTRODUCTION

A smile increases one's self-confidence in addition to enhancing one's physical attractiveness. The harmony of a beautiful smile is attributed to a multitude of characteristics, including lip position, tooth position and the morphology of the tooth in relation to the gingiva [1]. An appealing smile is also influenced by the condition, colour and shape of the gingival tissue. The colour of the gingiva is determined by the number of pigments such as melanin, melanoid, carotene and oxyhaemoglobin [2].

Melanin is found naturally in the skin, gingiva and the remaining portion of the oral mucous membrane. Melanocytes are a particular type of cell that produce this pigment [3]. Tyrosinase is an enzyme that catalyses the formation of melanin in specialised organelles called melanosomes.

Numerous treatment approaches have been developed to address hyperpigmented gingiva, as it appears to be a significant aesthetic concern. Additive techniques, such as free gingival grafting or the use of Acellular Dermal Matrix Allograft (ADMA) and subtractive treatment methods, such as conventional techniques (excision/scalpel or stripping surgical technique) [4], bur abrasion technique [5,6], are employed to remove the hyperpigmented gingival tissue during the periodontal surgical procedure known as gingival depigmentation. Various advanced procedures, including radiosurgery methods [7], cryosurgery [8], lasers [9,10] and electrosurgery [11], are also utilised. While these surgical techniques and lasers are commonly used to treat gingival hyperpigmentation, they have certain drawbacks. These operations often come with risks of recurrence, extensive postoperative wounds, discomfort and bleeding.

In order to reduce postoperative complications and recurrence rates, along with alleviating patient apprehensions, alternative treatment options are gaining popularity in recent times and can be explored. Nowadays, there is a growing demand for the use of natural products in the field of cosmetics and more people are willing to opt for them as they have fewer to no side-effects and are non toxic [12]. Phytochemicals derived from various plant sources have been tried for hyperpigmented lesions of the skin and have achieved great success [13]. Thus, understanding the benefits of natural and botanical extracts provides opportunities to develop new products to address pigmentation problems associated not just with the skin but also with the gingiva.

P-Coumaric acid (p-CA) is a common secondary metabolite found in plants. Coumaric acids are derivatives of cinnamic acid. It is present at significant levels in many fruits such as apples, pears, grapes, oranges, tomatoes and berries; in vegetables (e.g., beans, potatoes and onions); and in cereals (e.g., maize, oats and wheat) [14]. Previous literature has shown that p-CA, especially its conjugates, exhibits various bioactivities, including antioxidant, anti-inflammatory, anti-mutagenic, anti-ulcer, anti-platelet and anti-cancer activities, in addition to mitigating atherosclerosis, oxidative cardiac damage, Ultraviolet (UV)-induced damage to ocular tissues, neuronal injury, anxiety, gout and diabetes [14].

The p-CA is also found to be a potent anti-melanogenic agent. Its mechanism of action is attributed to the selective inhibition of the enzyme tyrosinase. Its anti-melanogenic effect has been demonstrated in various studies [15,16]. Another phytochemical,

quercetin, is a predominant flavanol-type flavonoid present in vegetables, fruits, beverages and medicinal plants. Consequently, quercetin has been extensively studied for its pharmacological benefits, including anti-inflammatory anti atherosclerotic, antioxidant and anti-cancer effects [17]. Quercetin has been found to have skin protective properties related to melanogenesis [18,19]. Thus, like p-CA, quercetin is also a selective inhibitor of the enzyme tyrosinase and consequently reduces melanin synthesis. However, to the authors knowledge, no study has been conducted on pigmented gingival tissues.

Considering the anti-melanogenic properties of these phytochemicals, the present study was planned to assess their effectiveness in reducing the melanin content of pigmented gingival tissue. The study aimed to evaluate the effectiveness of two phytochemicals, p-coumaric acid (3 µM) and quercetin (20 µM), in reducing the melanin content of pigmented gingival tissue and to compare the reduction in pigmentation with that of untreated pigmented gingival tissue.

MATERIALS AND METHODS

The present ex-vivo study was conducted at Bharati Vidyapeeth Deemed to be University, Dental College and Hospital, Pune, Maharashtra, India. The research proposal was approved by the Institutional Ethics Committee (IEC) to carry out the study (Regd. No.: EC/NEW/INST/2021/MH/0029).

Individuals visiting the study Institute were carefully selected based on the following inclusion and exclusion criteria.

Inclusion and Exclusion criteria: Systemically healthy subjects of either gender, aged between 18-45 years, with aesthetic concerns regarding physiologic gingival pigmentation, were included in the study. Subjects who had gingival pigmentation due to any other cause, such as smoking or drug use, or any systemic illnesses, such as diabetes or heart disease, as well as individuals with known allergies, pregnant and lactating females, or patients who were not willing to undergo the surgical depigmentation procedure, were excluded from the study.

All individuals fulfilling the above inclusion and exclusion criteria were explained the purpose and design of the study. Verbal and written informed consent was obtained from all participants willing to take part in the study.

Sample size calculation: The sample size was estimated using OpenEpi software (version 3.04):

- Confidence Interval (CI) 95% (2-sided) - 95%
- Alpha (Type 1 error) - 5%
- Beta (Type 2 error) - 20%
- Power - 80%
- Z value - 0.96
- Effect size - 20%
- Level of significance (usually 5%)

Based on the above data, a sample size of n=10 was derived. A total of 10 subjects with complaints of pigmented gingiva were selected for the study.

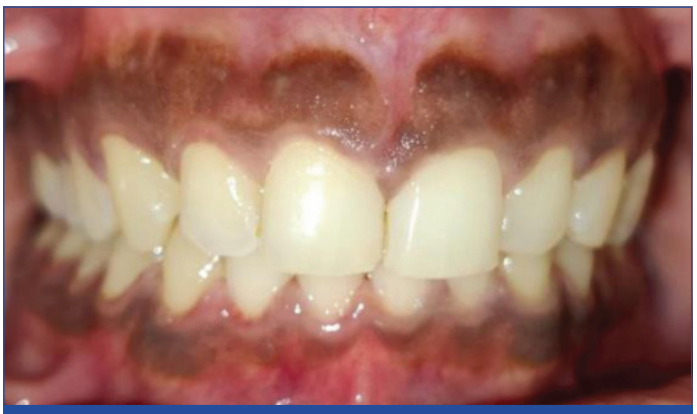
Study Procedure

A precise clinical examination was then carried out on a dental chair under standard lighting conditions, using a mouth mirror, explorer and graduated periodontal probe (University of North Carolina/ UNC-15). Subjects with pigmented gingiva were further assessed for their pigmentation score via visual examination using the Dummet Oral Pigmentation Index (DOPI) [20].

Score:

- 0: No clinical pigmentation (pink-coloured gingiva)
- 1: Mild clinical pigmentation (mild light brown colour)
- 2: Moderate clinical pigmentation (medium brown or mixed pink and brown colour)
- 3: Heavy clinical pigmentation (deep brown or bluish-black colour)

Subjects with a DOPI score of 2 were enrolled in the study [Table/Fig-1]. Phase I periodontal therapy was carried out for all participants willing to participate in the study. Blood investigations were also advised for all participants before they underwent the surgical depigmentation procedure.

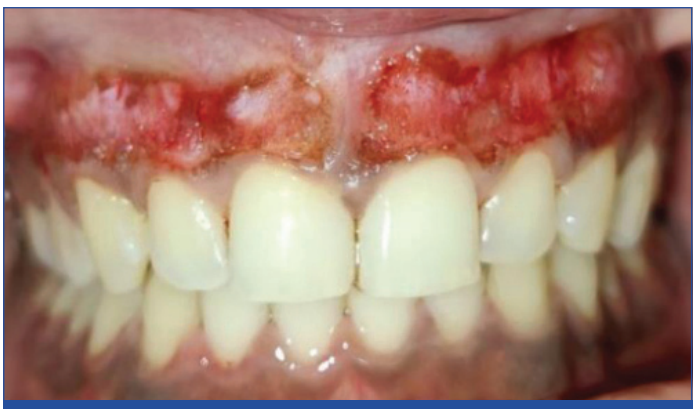


[Table/Fig-1]: Gingival hyperpigmentation DOPI

Participants were recalled at regular intervals to assess the status of gingival inflammation. They were scheduled for a depigmentation procedure after the gingival inflammation subsided.

Preparation of phytochemical solutions: The materials used for the preparation of the two phytochemical solutions were obtained commercially. P-Coumaric acid (p-CA) powder was dissolved in 10 mL of Dimethyl Sulfoxide (DMSO) to yield a solution with a concentration of 3 µM. Quercetin powder was dissolved in 10 mL of Dimethyl Sulfoxide (DMSO) to yield a solution with a concentration of 20 µM.

Surgical protocol [21]: The surgical procedure was carried out following all universal precautionary guidelines. Subjects were administered local anaesthesia (2% lignocaine with 1:200,000 adrenaline) using a 25-gauge needle via the local infiltration technique. After this, the hyperpigmented gingiva was excised using a scalpel [Table/Fig-2]. The excised pigmented gingiva was divided into three parts, each measuring 0.5×0.5 mm.



[Table/Fig-2]: Depigmentation done by scalpel.

These three parts were designated as separate groups:

- Group A: Control group
- Group B: P-coumaric acid (p-CA)
- Group C: Quercetin

Group A: The first part of the excised pigmented gingival tissue was used as a control, where none of the phytochemicals were applied. After excision, it was fixed immediately using freshly prepared 10% buffered formalin.

Group B: The second part of the excised pigmented gingival tissue was dipped in 1 mL of 3 µM p-CA for a period of 15 minutes [Table/Fig-3]. After a waiting period of 15 minutes, the tissue was washed with fresh saline and then fixed using 10% buffered formalin.

Group C: The third part of the excised pigmented gingival tissue was dipped in 1 mL of 20 µM Quercetin for a period of 15 minutes [Table/Fig-4]. After a waiting period of 15 minutes, the tissues were washed with fresh saline and then fixed using freshly prepared 10% buffered formalin. The tissue samples were then sent for histological analysis.

[Table/Fig-3]: Excised tissue dipped in 3 µM p-Coumaric acid (p-CA).
[Table/Fig-4]: Excised tissue dipped in 20 µM Quercetin. (Images from left to right)

Tissue preparation for histochemistry: The specimens were dehydrated in ascending grades of ethyl alcohol (95%, 99%, 100%), then cleared in xylene and embedded in paraffin. Sections of 4-5 µm in thickness were obtained and collected on positively charged microscope slides. Tissue sections were deparaffinised using xylene and then rehydrated using distilled water before histological staining and immunolabeling.

Histological staining: Tissue sections from all three groups were stained with H&E stain. They were then examined using a light microscope at 40X magnification, equipped with a digital camera.

Immunohistochemical examination [22]: In the study, the immunohistochemical examination was carried out using a "polyclonal anti-Human Melanoma Black 45 (anti-HMB-45)" antibody.

The tissue sections were incubated in Phosphate Buffered Saline (PBS) for 10 minutes at 37°C. After two washes with PBS, the unmasking of the antigens was carried out. The antigen (melanocyte) was unmasked by boiling the tissue section in a citrate buffer (pH 6.0) using a microwave oven for three cycles of five minutes each, with a one-minute break in between the cycles. The sections were incubated with the primary antibodies, specifically polyclonal anti-PBS 45 (anti-HMB-45), overnight at 4°C. The following day, after washing in PBS, the sections were incubated with a secondary universal antibody. The substrate DAB (3,3'-Diaminobenzidine) was applied until the desired brown colour developed (2-10 minutes). Finally, the sections were counter-stained with Mayer's haematoxylin for 30 seconds to visualise the tissue topography.

The melanocytes containing the melanin granules were visualised at a magnification of 400X. The density and distribution of melanin granules were determined by measuring the density of melanin granules based on the scale provided by Patsakas A et al., (1981) [23].

- Score 0: Absence of melanin granules
- Score 1: Rare and scattered melanin granules
- Score 2: Dense but not aggregated melanin granules
- Score 3: Dense and aggregated melanin granules

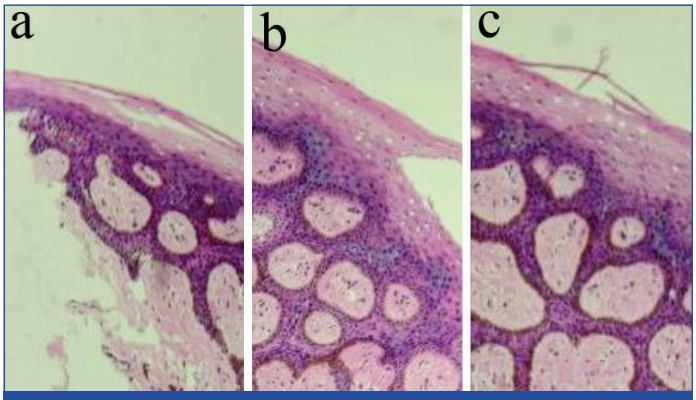
The scores of melanin pigmentation in the excised gingival tissue treated with the experimental phytochemicals (Groups B and C) and those of untreated tissue (Group A) were noted for each group.

STATISTICAL ANALYSIS

The data obtained was subjected to statistical analysis. SPSS version 20.0 was used for analysing the data. Descriptive data were presented in tables using numbers and percentages. Intergroup comparisons of the study variables were performed using the t-test and post-hoc Tukey's test. For the above tests, a p-value ≤0.05 was considered statistically significant.

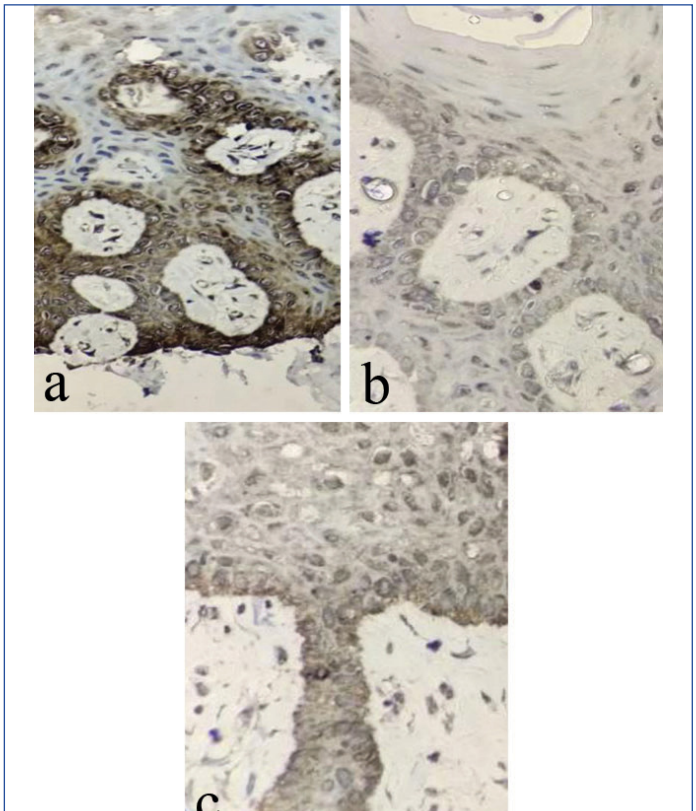
RESULTS

The slides prepared with H&E staining were observed under 40x magnification. Upon examination, all four layers of the gingival epithelium- namely the stratum basale, stratum spinosum, stratum granulosum and stratum corneum- were identified [Table/Fig-5a-c]. All cell layers exhibited intact cellular structures, with no alterations in cell shape, size, or position across all three groups. The nuclei in all cell layers displayed normal shape, size and positioning in all three groups.



[Table/Fig-5]: a) Slide of untreated pigmented gingival tissue (H&E slide, 40x); b) Slide of pigmented gingival tissue treated with 3 µM of p-Coumaric acid (p-CA) (H&E slide, 40x); c) Slide of pigmented gingival tissue treated with 20 µM of Quercetin (H&E slide, 40x).

For a detailed assessment of melanocytes and the degree of pigmentation, immunohistochemical analysis was conducted using the HMB-45 antibody. [Table/Fig-6a-c] shows the stained slides for the immunohistochemical assay across all three groups at a magnification of 400X.



[Table/Fig-6]: a) HMB-45 immunohistochemical stained slide of untreated pigmented gingival tissue; b) HMB-45 immunohistochemical stained slide of tissue treated with 3 µM p-Coumaric acid; c) HMB-45 immunohistochemical stained slide of tissue treated with 20 µM Quercetin.

The reduction in melanin content scores was compared between untreated tissue and tissue treated with the two phytochemicals. The percentage of samples with varying melanin content scores (HMB-45 antibody immunoreaction) for all three groups: Group-A (Control Group), Group-B (3 µM p-CA) and Group-C (20 µM Quercetin) is illustrated in [Table/Fig-7].

Group-A (Control group)	
Score	**No. of samples (%)**
0	0
1	0
2	0
3	10 (100)
Group-B {P-Coumaric acid (p-CA)}	
Score	**No. of samples (%)**
0	0
1	7 (70)
2	2 (20)
3	1 (10)
Group-C (Quercetin)	
Score	**No. of samples (%)**
0	0
1	0
2	7 (70)
3	3 (30)

[Table/Fig-7]: Percentage of samples with different scores (Patsakas A et al., 1981) [23] in all the three groups.

The comparison of the mean melanin content scores for all three groups: Group-A (Control Group), Group-B (3 µM p-CA) and Group-C (20 µM Quercetin) is depicted in [Table/Fig-8]. The mean melanin content score for Group-A was 3±0.0, while for Group-B it was 1.3±0.45. There was a statistically significant difference between both groups (p-value <0.05), with Group-A exhibiting a higher score compared to Group-B, indicating that Group-B showed less melanin content than Group-A.

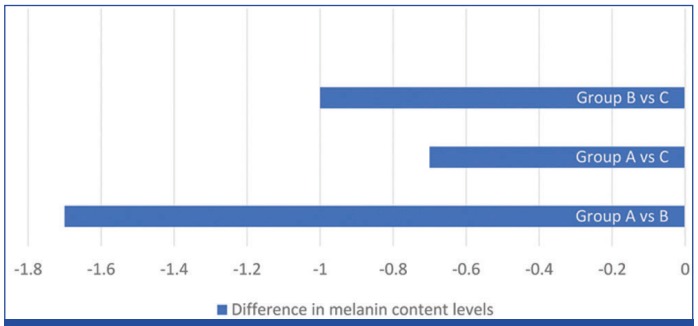
Comparison of Group-B {P-Coumaric acid (p-CA)} with Group-A (Control Group)				
Groups	**Total sample (n)**	**Mean value**	**Standard Deviation (SD)**	**p-value (sig.)**
Group-A	10	3.0	0	p<0.001** (<0.05*)
Group-B	10	1.3	0.45	
Comparison of Group-C (Quercetin) with Group-A (Control Group)				
Groups	**Total sample (n)**	**Mean value**	**Standard Deviation (SD)**	**p-value (sig.)**
Group-A	10	3.0	0	p<0.001** (<0.05*)
Group-C	10	2.3	0.45	
Comparison of Group-B {P-Coumaric acid (p-CA)} with Group-C (Quercetin)				
Groups	**Total sample (n)**	**Mean value**	**Standard Deviation (SD)**	**p-value (sig.)**
Group-B	10	1.3	0.45	p<0.001** (<0.05*)
Group-C	10	2.3	0.45	

[Table/Fig-8]: Intergroup comparison of different groups for mean melanin content Score (Tukey's post-hoc test).

The mean melanin content score for Group-A was 3±0.0 and for Group-C, it was 2.3±0.45. There was a statistically significant difference between both groups (p-value <0.05), with Group-C showing less melanin content compared to Group-A.

The mean melanin content score for Group-B was 1.3±0.45, while for Group-C it was 2.3±0.45. There was a statistically significant difference between both groups (p-value <0.05), with Group-C exhibiting more melanin content compared to Group-B.

The differences in mean melanin content scores among the three groups is shown in [Table/Fig-9]. The difference was greatest between Group-A and Group-B (-1.7), followed by Group-A and Group-C (-0.7) and Group-B and Group-C (-1). This indicates that a higher reduction in melanin content was observed with Group-B (p-CA) than with Group-C (Quercetin) when compared to the control samples.



[Table/Fig-9]: Mean difference between the mean melanin content scores (Tukey's post-hoc analysis).

DISCUSSION

The study was conducted to evaluate the depigmenting effect of 3 µM of p-CA and 20 µM of Quercetin on excised pigmented gingival tissue. The reduction in melanin content was compared with untreated tissue and also among the tissues treated with the two phytochemicals using immunohistochemical analysis with the HMB-45 antibody. Both plant chemicals tested showed a reduction in melanin content.

Among the various herbal products, p-CA and quercetin have been found to be effective in treating hyperpigmentation lesions [15,16,18,19]. Their anti-melanogenic properties have been attributed to their ability to inhibit the enzyme tyrosinase, which is the rate-limiting enzyme in melanogenesis. p-CA has also been found to downregulate the activity of α-melanocyte-stimulating hormone [24,25], which further inhibits cellular melanogenesis.

Both p-CA and quercetin exhibit antioxidant properties due to their ability to scavenge free radicals and reduce reactive oxygen species [26]. Additionally, quercetin has been shown to protect cells from oxidative damage by increasing intracellular antioxidant levels and reducing lipid peroxidation [27]. It has also been found to protect melanocytes against H_2O_2-induced oxidative damage [28].

Owing to the aforementioned properties, it is confirmed that both p-CA and quercetin are potent inhibitors of melanogenesis and can be effectively employed as depigmenting agents.

Different concentrations of these phytochemicals have been studied [24,25] for their anti-melanogenic effects. In the present study, a lower concentration of p-CA (3 µM) has been used and found to be effective in reducing melanin content. Similarly, Kim A et al., (2011) also found tyrosinase inhibition at a concentration of 3 µM [29]. This concentration has also been found to be effective in reducing melanin content in cell cultures of human epidermal melanocytes [24].

Studies have been conducted to assess cellular toxicity caused by p-CA [24,30]. All these studies showed that, at various concentration ranges for the inhibition of cellular melanogenesis, no significant levels of toxicity were noted. In the present study, microscopic examination also revealed no cellular damage. Therefore, the concentrations that demonstrate melanin inhibition can be safely used without damaging the tissues.

In relation to the phytochemical quercetin, various concentrations have been tested for their inhibition of melanogenesis. Arung ET et al., found that quercetin at a concentration of less than 100 µM is able to inhibit melanin content in B16 melanoma cells [31]. However, according to Yang YM et al., who evaluated the effect of

quercetin at different concentrations on B16 melanoma cells and normal human epidermal melanocytes [32], quercetin was able to reduce melanin content (tyrosinase activity) in a dose-dependent manner at concentrations above 10 µM. In the concentration range of 5-10 µM, there was an increase in tyrosinase activity, whereas a reduction in activity was observed in the 20-50 µM range. Additionally, a reduction in the activity of Tyrosinase Related Protein-1 (TRP-1) and TRP-2 was noted at 20-50 µM. Based on these findings, a concentration of 20 µM was selected for the present study.

However, Nagata H et al., tested quercetin at concentrations of 1, 5, 10 and 20 µM on normal human epidermal melanocytes and human melanoma cells and found an increase in melanin content and enhanced tyrosinase activity [33]. Similar results were demonstrated by Takeyama R et al., [34], where quercetin was tested on a human skin model, Melanoderm, in the concentration range of 10-100 µM. Takekoshi S et al., also demonstrated an increase in tyrosinase activity and a subsequent increase in melanin content in human melanoma cells when treated with quercetin in the range of 1-20 µM [35]. These findings indicate that the anti-melanogenic effect of quercetin is dose- and cell-specific.

Even though there are studies [31-33] that indicate a 20 µM concentration of quercetin leads to an increase in tyrosinase activity and subsequent increase in melanin content, the present study has exhibited a positive reduction in melanin content at the 20 µM concentration of quercetin, based on pair-wise comparison using Tukey's post-hoc analysis.

Various studies have been carried out regarding the cellular toxicity caused by quercetin. Yang YM et al., demonstrated that quercetin at a concentration above 20 µM exhibited cellular toxicity [32]. Similarly, Takekoshi S et al., reported no cellular toxicity below 20 µM [35]. However, another study [26] reports cellular toxicity at concentrations above 10 µM. Taking this into consideration, the present study was planned accordingly, wherein light microscopic examination was conducted to rule out any cellular damage prior to immunohistochemical analysis. On light microscopic examination, no cellular damage was noted when tissue samples were treated with quercetin at a concentration of 20 µM in the present study.

From the present study, it can thus be concluded that both phytochemicals can reduce cellular melanin content at the specified concentrations without causing any cellular damage.

Limitation(s)

The study was carried out with certain limitations. Only one particular concentration of both phytochemicals was studied. To gain a better understanding of their effects on melanogenesis, a range of concentrations should be employed. The present study was conducted on mildly pigmented lesions, while heavily pigmented lesions were examined at different concentrations. Higher concentrations need to be tested for their cellular toxicity.

CONCLUSION(S)

Hence, regarding the properties and derivations from the results of the present study, p-CA and quercetin may be considered potential depigmenting agents that could be further developed for intraoral use to treat gingival pigmentation.

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