

COMPARATIVE STUDY OF ANTIOXIDANT, ANTI-INFLAMMATORY, AND ANTIMICROBIAL ACTIVITIES OF LAC DYE AND D&C RED No. 27

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Abstract

Lac dye and D&C red No. 27, natural and synthetic dye, are commonly used in pharmaceuticals and cosmetics. Their antioxidant, anti-inflammatory, and antimicrobial activities were investigated using the DPPH, the thermal inhibition of egg albumin protein denaturation and the agar diffusion methods, respectively. Lac dye demonstrated stronger antioxidant activity than ascorbic acid (the positive control) and D&C red No. 27, with EC₅₀ values of 2.03±0.08, 8.28±0.02, and 397.87±1.16 µg/ml, respectively. D&C red No. 27 exhibited anti-inflammatory activity (EC₅₀ value 114.61±8.46 µg/ml) higher than diclofenac sodium (EC₅₀ 754.11±1.42 µg/ml), whereas lac dye induced inflammation with an IC₅₀ value of 136.40±2.31 µg/ml. At a concentration of 0.25% w/v, lac dye effectively inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Porphyromonas gingivalis*, producing clear inhibition zones of 17.7±0.6, 18.3±0.6, and 24.3±0.6 mm, respectively. However, lac dye displayed antifungal activity against *Candida albicans* and *Candida tropicalis* compared to the solvent (PEG 400) at a concentration of 2% w/v. D&C red No. 27 inhibited against *S. aureus*, *E. coli*, and *P. gingivalis*, with clear zones of 15.3 ± 0.6, 15.3 ± 0.6, and 22.3 ± 0.6 mm, respectively, at a concentration of 0.25%. Notably, D&C red No. 27 demonstrated strong antimicrobial activity against *Propionibacterium acnes*, with an inhibition zone of 41.0±1.0 mm, which surpassed the 24.0±1.7 mm zone produced by lac dye at the same concentration of 0.25% w/v. However, D&C red No. 27 exhibited only a limited ability to inhibit both types of fungi. These findings suggest that both dyes may offer valuable benefits for the development of future formulation.

Keywords: Lac dye; D&C red no.27; Antioxidant Activity; Anti-Inflammation; Anti-Microbial Activity

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Introduction

Lac dye and D&C red No. 27 dye are red dyes commonly used in pharmaceuticals, cosmetics, and some countries are used as coloring agent in food (Sharma *et al.*, 2020; U.S. Food and Drug Administration, 2022).

Lac dye is used in various food products, including fruit beverages, vegetable juice (pulp), fruit-flavored drinks, soda, compound seasonings, jam, cocoa products, bakery fillings, imitation wine, chocolates, and chocolate-based products (Sharma *et al.*, 2020). Additionally, it is applied in fabric dyeing and cosmetics (Chairat, 2004; Jimtaisong, 2020). Lac dye is a natural dye produced by lac insects of the *Kerria* spp. (Tachardiidae family; Homoptera order), which feed on the sap of specific host trees. This secretion is believed to help shield the insects from harmful UV radiation. The dye is sourced from stick lac, with laccaic acid being the main coloring component. To create the refined product known as seedlac, the scraped lac is crushed and washed to achieve a consistent size. During this process, the lac dye becomes soluble in water, which is then processed to obtain crystallized dye. The red hue of the resin originates from the insects. While lac dye is derived during the washing of stick lac, the pure dye is only minimally soluble in water. The primary colorant, laccaic acid, is a hydroxyanthraquinone carboxylic acid. Lac dye contains at least five closely related pure compounds, all of which are anthraquinone derivatives, designated as laccaic acid A, B, C, D, and E (Figure 1) (Sharma *et al.*, 2020). Lac dye exhibited radical scavenging activity, as demonstrated through testing with DPPH and ABTS assays. At a certain concentration, lac dye demonstrated a scavenging rate of 68% for DPPH[•] radicals and 87% for ABTS^{•+} radicals. Its antioxidant capacity was 0.20 times that of ascorbic acid in relation to DPPH[•], but 3.42 times greater when it came to ABTS^{•+}, indicating its varying effectiveness depending on the radical type (Zhang *et al.*, 2011). Lac dye had shown anti-inflammatory properties by suppressing the ERK/NFκB inflammatory signaling pathway, leading to a decrease in the expression of inflammatory cytokines such as TNFα, IL-1β, and IL-6 (Kabeer *et al.*, 2023). There are few researches available on the antimicrobial effects of lac dye or its key components against human pathogens such as *Staphylococcus aureus* and *Escherichia coli* (Dey and Ghosh, 2018; Do *et al.*, 2023). Moreover, it has been found that methylated lac dye can inhibit plant pathogenic fungi (Srivastava *et al.*, 2017).

However, there remains limited information on its antimicrobial activity.

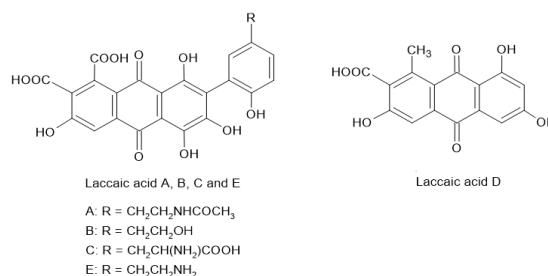


Figure 1. Chemical structures of laccaic acid A, B, C, D and E (Sharma *et al.*, 2020)

D&C red No. 27, also known as Phloxine O (Colour Index No. 45410:1), is a synthetic xanthene dye primarily made up of 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein. Its production involves multiple stages: first, 3,4,5,6-tetrachlorophthalic acid (TCPA) or its anhydride reacts with two equivalents of resorcinol, producing tetrachlorofluorescein. This intermediate undergoes partial purification and is then brominated to form the main component of D&C red No. 27. Alkaline hydrolysis of this compound yields its sodium salt form, which is known as D&C red No. 28 or Phloxine B (Colour Index No. 45410). Both dyes are certified by the U.S. for use as color additives in pharmaceuticals and cosmetics, according to the Code of Federal Regulations (CFR). Additionally, D&C red No. 28 is approved for use in food products in Japan, where it is labeled as Food red No. 104. Figure 2 provides an overview of the chemical structure and synthesis process for D&C red No. 27 and D&C red No. 28 (Weisz and Krantz, 2014). Previous studies have provided only limited insights into the antioxidant and anti-inflammatory properties of D&C red No. 27 dye. Specific data regarding its antioxidant and oxidative stress effects remain unavailable, with existing research focusing primarily on phloxine dye. In studies using Chinese hamster ovary cells, phloxine dye showed no significant changes in malondialdehyde or catalase activity compared to the control group ($p > 0.05$). However, it significantly decreased glutathione levels and enhanced glutathione peroxidase activity ($p < 0.05$). These findings indicate that phloxine dye may induce oxidative stress in certain parameters (Demirkol *et al.*, 2020). Regarding its anti-

inflammatory activity, evidence revealed that D&C red No. 27 significantly lowered the production of thymic stromal lymphopoietin (TSLP), an interleukin 7-like cytokine responsible for activating dendritic cells to initiate Th2 inflammatory immune responses. This effect was observed in PAM212 cells, where inflammation had been induced with 30 nM phorbol 12-myristate 13-acetate, and in KCMH-1 cells, a squamous cell carcinoma line derived from mouse keratinocytes ($p < 0.05$). This reduction occurred when dye concentrations of 10, 20, and 50 μM were applied, showing a clear dose-response relationship. In an experiment investigating the alleviation of acute dermatitis symptoms in mouse ear skin inflamed by calcipotriol, D&C red No. 27 demonstrated significant anti-inflammatory properties. The dye markedly reduced the levels of TSLP, histamine, IgE, IL-4, IL-5, IL-13, and the expression of Ox40 ligand—a member of the tumor necrosis factor ligand family, which plays a role in T cell stimulation and differentiation—when compared to the control group (Lee *et al.*, 2018). From the literature review, there is no available research on the antimicrobial properties of D&C red No. 27.

Due to the widespread use of both dyes and the limitations of available data, this research aimed to compare their antioxidant activity using the DPPH assay, their anti-inflammatory effects by reducing heat-induced denaturation of egg white proteins, and their antibacterial activity against some human pathogenic strains. The findings will contribute to more informed decisions regarding the use of these two dyes.

Materials and Methods

Materials

Lac dye and D&C red No. 27 dye were purchased from Creasia Mill Co., Ltd., Lampang, Thailand, and Sun Chemical Corporation, Waterview Boulevard, Parsippany, NJ, USA, respectively. The antioxidants 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid were obtained from Sigma-Aldrich®, St. Louis, USA, and Fisher Scientific, Loughborough, UK, respectively. Phosphate-buffered saline (PBS) was prepared using sodium chloride (Ajax Finechem, Thermo Fisher Scientific, Auckland, New Zealand), sodium dihydrogen orthophosphate (Ajax Finechem, Thermo Fisher Scientific, Auckland, New Zealand), and disodium hydrogen phosphate anhydrous (QR&C™, New Zealand). Dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG400) were sourced from Loba Chemie Pvt. Ltd., Mumbai, India. Methanol was acquired from RCI Labscan Limited, Bangkok, Thailand. The spectrophotometer (Hitachi U-2000 spectrophotometer, Tokyo, Japan) was used to determine the antioxidant and anti-inflammatory activities. For the antibacterial study, tryptic soy agar and broth (Difco™, Detroit, MI, USA), sheep blood agar (Ministry of Public Health, Nonthaburi, Thailand), and an anaerobic jar (Forma Anaerobic System, Thermo Scientific, Ohio, USA) were utilized. For the antifungal study, Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) (Difco, Detroit, MI, USA) were used.

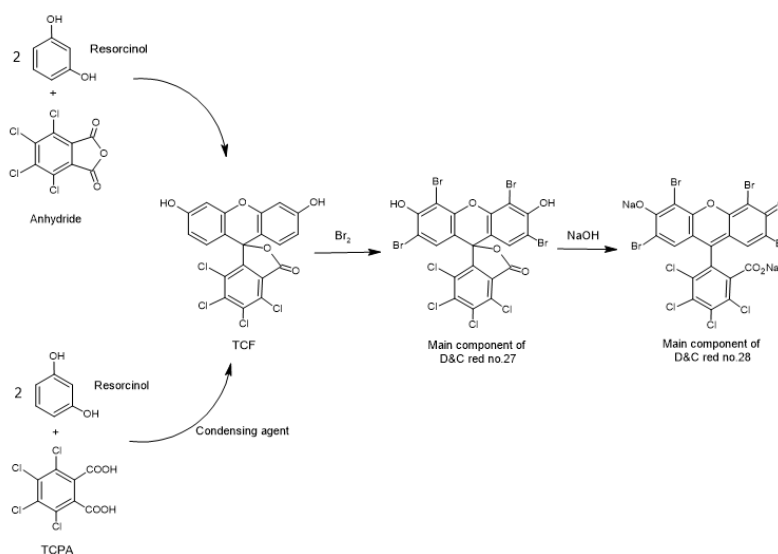


Figure 2. Outlines the preparation of D&C red No. 27 and 28 through the condensation of 3,4,5,6-tetrachlorophthalic acid (TCPA) or its anhydride with resorcinol, followed by the bromination of the resulting condensation product, 4,5,6,7-tetrachlorofluorescein (TCF) (Weisz and Krantz, 2014)

Staphylococcus aureus ATCC 6538, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231 were obtained from the Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. *Propionibacterium acnes* ATCC 14916 and *Porphyromonas gingivalis* ATCC 33277 were provided by Thai Can Biotech Co., Ltd., Bangkok, Thailand. *Candida tropicalis* TISTR 5306 was sourced from the Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand. Gentamicin sulfate (GS) and fluconazole (Fc) were generously supplied by the Government Pharmaceutical Organization, Bangkok, Thailand. Whatman® Antibiotic Assay Discs were obtained from Merck Ltd.

Determination of the antioxidant activity

This activity was evaluated using the DPPH assay. The process was done according to the reference (Khin, 2021). The reaction involved mixing 75 µg/ml of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) in methanol with varying concentrations of the sample solution in a 1:1 ratio. The mixture was kept at room temperature in the dark for 1 h. Afterward, the absorbance was measured at 517 nm using a UV spectrometer, with the solvent serving as the control and the sample as the blank. Each test was conducted in triplicate. The percentage of antioxidant activity (%AA) was calculated using the following equation.

$$\% AA = \frac{(AC - (AS - ASB))}{AC} \times 100$$

Where *AC* is absorbance of control, *AS* is absorbance of sample, and *ASB* is absorbance of sample blank.

The half maximal effective concentration (EC₅₀ value), which expressed the antioxidant activity, was calculated using the equation derived from the data. This was achieved by plotting the results in an Excel program to obtain a graphical representation of the relationship between concentration and antioxidant activity, allowing for the determination of the EC₅₀ value.

Determination of the anti-inflammatory activity

Thermal inhibition of the egg albumin protein denaturation method was used to test this activity. The procedure for conducting the lab was based on the research by Chaiya *et al.* 2022. The reaction comprised 0.2 ml of white egg, 2.8 ml of phosphate buffer saline (PBS) pH 6.4, and sample 2 ml. The water used in the experiment was double-distilled water. The mixture was incubated at 37°C for 15 minutes, then heated in a water bath at 70°C for 5 minutes. The turbidity was subsequently

measured at 660 nm using a UV spectrometer. The solvent was used as a control and the sample using PBS instead of egg white was blank. The control group was 50%v/v DMSO in water as solvent. The test was determined in triplicate. The %anti-inflammation (%AI) was calculated as an equation.

$$\%AI = \frac{(TC - (TS - TSB))}{TC} \times 100$$

Where *TC* is the turbidity of control, *TS* is turbidity of sample, and *TSB* is turbidity of sample blank.

The half maximal effective concentration (EC₅₀ or IC₅₀ value), which indicates the anti-inflammatory activity, was determined by plotting the results in an Excel program to derive the necessary calculations.

Determination of the antimicrobial activity

The antimicrobial activity of both dyes was evaluated using the agar cup diffusion method at concentrations of 0.25%, 1%, 2%, and 4% w/v, with polyethylene glycol 400 (PEG400) as the solvent. Standard microbial strains were selected to assess antibacterial and antifungal activity. The antibacterial activity was tested against *S. aureus*, *E. coli*, *P. acnes*, and *P. gingivalis*, while antifungal activity was evaluated against *C. albicans* and *C. tropicalis*. The experimental procedure began with the cultivation of *S. aureus*, *E. coli*, and *P. gingivalis* in Tryptic Soy Broth for 18-24 h. *P. acnes* was cultured in Brain Heart Infusion broth for 48-72 h. *C. albicans* and *C. tropicalis* were grown in Sabouraud Dextrose Broth for 18-24 h. All microorganisms were incubated at a temperature of 35-37°C. Following incubation, bacterial cultures (*S. aureus*, *E. coli*, *P. gingivalis*, and *P. acnes*) were diluted with normal saline solution to match the turbidity of the 0.5 McFarland standard. Fungal cultures (*C. albicans* and *C. tropicalis*) were adjusted to the 2 McFarland standard using normal saline. The prepared microbial suspensions were inoculated onto agar plates appropriate for each microorganism. *S. aureus* and *E. coli* were plated on Tryptic Soy Agar, *P. gingivalis* on Blood Agar, *P. acnes* on Brain Heart Infusion Agar, and *C. albicans* and *C. tropicalis* on Sabouraud Dextrose Agar. Once the plates were prepared, 100 µL sample of each test solution was applied to a cup placed on the agar surface. Additionally, discs containing the positive control compounds were placed on inoculated plates. The plates were then incubated under conditions suitable for each microbial strain. Anaerobic bacteria (*P. gingivalis* and *P. acnes*) were incubated in an anaerobic jar to ensure appropriate growth conditions. Positive control

discs were prepared by dissolving gentamicin sulfate (GS) and fluconazole (Fu) in ethanol at a concentration of 8 and 20 mg/ml, respectively. Each solution was applied to a 6 mm blank disc and dried to ensure a final content of 20 and 50 µg per disc for GS and Fu, respectively. These discs were used as positive controls for antibacterial and antifungal testing, respectively. Antimicrobial activity was determined by measuring the diameter of the inhibition zone (n=3) around the test sample or control discs.

Results and Discussion

Antioxidant activity

The free radical scavenging activity was evaluated using the DPPH assay. All tested samples exhibited antioxidant activity, with lac dye showing the strongest effect, followed by ascorbic acid and D&C red No. 27, as indicated by their EC₅₀ values of 2.03 ± 0.08 , 8.28 ± 0.02 , and 397.87 ± 1.16 µg/ml, respectively. Lac dye demonstrated superior antioxidant activity compared to ascorbic acid, which was used as a positive control. The results are presented in Figure 3. In previous research, lac dye and ascorbic acid were found to have EC₅₀ values of 12.26 µg/ml and 4.52 µg/ml, respectively, when measured using the same DPPH assay (Jimtaisong *et al.*, 2013). Based on the other previous findings, it can be suggested that the source of lac dye and ascorbic acid may influence their antioxidant activity. The antioxidant effect of lac dye observed in this study aligns with another study where lac dye was shown to effectively scavenge DPPH[•] and ABTS^{•+} radicals but was unable to scavenge O₂^{•-} radicals. This consistency supports the idea that lac dye has selective free radical scavenging abilities depending on the type of radical (Zhang *et al.*, 2011). The antioxidant activity of lac dye is likely attributed to its anthraquinone compounds, particularly laccic acids A-E, which are the important components of lac dye. Various types of anthraquinones and their derivatives have been previously reported to possess significant antioxidant properties, suggesting that these compounds play a key role in the radical scavenging activity observed in lac dye (Zhao and Zheng, 2023). Regarding D&C red No. 27 dye, which belongs to the phloxine group, no results from the DPPH assay and radical scavenging have been reported. However, the studies have shown that phloxine dye significantly reduced glutathione levels ($p \leq 0.05$) and significantly increased glutathione peroxidase activity ($p < 0.05$) in Chinese hamster ovary cells when compared to the control group. This suggests that while its direct

antioxidant activity via DPPH scavenging is unknown, phloxine dye may influence oxidative stress by altering cellular antioxidant defense mechanisms (Demirkol *et al.*, 2020). From previous studies, it has been noted that D&C red No. 27 induces oxidative stress. However, in this study, D&C red No. 27 demonstrated some radical scavenging activity. This indicates a potentially complex interaction where, despite its known role in promoting oxidative stress, D&C red No. 27 may also possess the ability to scavenge free radicals under certain conditions.

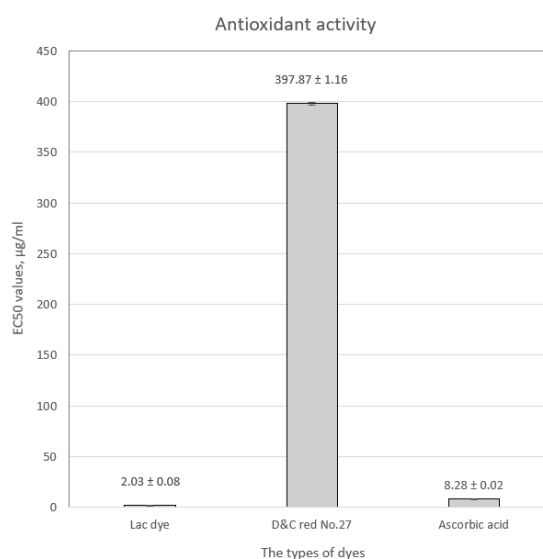


Figure 3. Antioxidant activity of lac dye, D&C Red No. 27, and ascorbic acid, expressed as EC₅₀ values using the DPPH method (n=3)

Anti-inflammatory activity

Chronic inflammatory disorders, such as protein glycation phenomena observed in conditions like cataracts and Alzheimer's disease, often originate with the denaturation of proteins (Saso *et al.*, 2001). To assess this activity, thermal inhibition of egg albumin protein denaturation was conducted. Protein denaturation typically results from alterations in the quaternary, tertiary, and secondary structures, which disrupt the protein's shape and lead to a loss of its biological function. This deformation is usually caused by physical factors such as heat or radiation, or by chemical agents like acids and alkalies. Several forces, including hydrogen bonds, ionic interactions, disulfide bridges, and hydrophobic interactions, are responsible for maintaining the protein's native structure. These covalent and non-covalent forces stabilize the protein. If a tested substance disrupts

these forces, it may influence the denaturation of the protein (Devlin, 1992). Interpretation of the results suggests that a substance's ability to reduce the denaturation of egg white protein by heat (indicated by decreased turbidity compared to the control group) signifies an anti-inflammatory effect. Conversely, if a substance causes more denaturation (indicated by increased turbidity), it implies a pro-inflammatory effect. From the data in Table 1, lac dye exhibited a negative percentage of anti-inflammatory activity, while D&C red No. 27 and diclofenac sodium (the positive control) showed positive percentages. This indicates that lac dye caused inflammation, with an IC_{50} value of 136.40 ± 2.31 $\mu\text{g/ml}$, whereas D&C red No. 27 and diclofenac sodium demonstrated anti-inflammatory activity, with EC_{50} values of 114.61 ± 8.46 $\mu\text{g/ml}$ and 754.11 ± 1.42 $\mu\text{g/ml}$, respectively.

Notably, D&C red No. 27 displayed greater anti-inflammatory activity than the positive control. Lac dye's pro-inflammatory effect may be attributed to its high content of laccic acids, which contain multiple hydroxyl and carboxylic functional groups. These functional groups can disrupt hydrogen bonds, one of the key forces that help maintain a protein's three-dimensional structure. As a result, the protein becomes more prone to denaturation (Malik *et al.*, 2016). Although lac dye did not exhibit anti-inflammatory properties through the inhibition of protein denaturation, it demonstrated anti-inflammatory effects by reducing the expression of inflammatory cytokines, specifically $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 . This suggests

that lac dye may exert its anti-inflammatory activity through a different biochemical pathway, influencing cytokine regulation rather than directly protecting proteins from denaturation (Kabeer *et al.*, 2023). This finding aligns with a previous study that demonstrated lac dye's ability to inhibit the expression of key pro-inflammatory genes, including COX-2 , iNOS , IL-6 , $\text{TNF-}\alpha$, and PGE_2 , when tested using the macrophage cell line RAW 264.7. This further supports the idea that lac dye exerts its anti-inflammatory effects by modulating the expression of inflammation-related genes rather than through protein stabilization (Sunantawanich, 2021). Currently, no information is available on the anti-inflammatory properties of D&C red No. 27 or other xanthene dyes related to the inhibition of protein denaturation, as demonstrated in this research. However, D&C red No. 27 has been shown to exhibit anti-inflammatory activity through a different mechanism. It suppresses thymic stromal lymphopoietin (TSLP), an interleukin 7-like cytokine, in both murine keratinocyte cell lines and a murine acute dermatitis model induced by calcipotriol. Furthermore, it decreases the production of histamine, IgE, IL-4 , IL-5 , IL-13 , and reduces the expression of the Ox40 ligand. This indicates that D&C red No. 27 may mediate its anti-inflammatory effects by modulating cytokine activity and downregulating the expression of certain members of the tumor necrosis factor ligand family to exert its anti-inflammatory effects (Lee *et al.*, 2018).

Table 1. Anti-inflammatory activity by thermal inhibition of egg albumin protein denaturation method of lac dye, D&C red No. 27, and diclofenac sodium (n=3)

Samples	Final concentration ($\mu\text{g/ml}$)	%Anti-inflammation			Mean $\pm$ S.D.
		n1	n2	n3	
Lac dye	25	-18.22	-19.57	-18.52	-18.77 $\pm$ 0.71
	50	-25.73	-25.88	-26.18	-25.93 $\pm$ 0.23
	100	-39.89	-39.29	-38.84	-39.34 $\pm$ 0.53
	150	-55.21	-53.55	-53.70	-54.15 $\pm$ 0.91
	200	-67.32	-66.12	-70.02	-67.82 $\pm$ 2.00
IC_{50} value, $\mu\text{g/ml}$ (induced inflammation)		135.81	138.94	134.44	136.40 $\pm$ 2.31
D&C red no.27	15.63	22.00	19.09	16.18	19.09 $\pm$ 2.91
	31.25	25.28	22.15	22.15	23.19 $\pm$ 1.81
	125	53.17	50.71	53.17	52.35 $\pm$ 1.42
	250	68.46	66.00	66.22	66.89 $\pm$ 1.36
	500	72.71	71.59	72.93	72.41 $\pm$ 0.72
EC_{50} value, $\mu\text{g/ml}$ (anti-inflammation)		105.02	120.99	117.83	114.61 $\pm$ 8.46
Diclofenac sodium	600	38.73	38.90	38.47	38.70 $\pm$ 0.22
	700	46.87	46.96	46.62	46.82 $\pm$ 0.18
	800	52.50	52.93	52.50	52.64 $\pm$ 0.25
	900	59.90	60.59	60.67	60.38 $\pm$ 0.42
	1000	68.33	67.47	67.64	67.81 $\pm$ 0.46
	1100	75.56	75.39	75.47	75.47 $\pm$ 0.09
	1200	82.36	82.70	82.27	82.44 $\pm$ 0.23
EC_{50} value, $\mu\text{g/ml}$ (anti-inflammation)		754.44	752.56	755.34	754.11 $\pm$ 1.42

Table 2. Inhibition zone diameters of different concentrations of lac dye solutions (Lac) and D&C red No. 27 (DC) in PEG 400 at concentration 0.25, 1, 2, 4%w/v against *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *P. gingivalis* ATCC 33277, *P. acnes* ATCC 14916, *C. albicans* ATCC 10231 and *C. tropicalis* TISTER 5306 (n=3)

Formulation	Inhibition zone $\pm$ S.D. (mm)					
	<i>S. aureus</i> ATCC 6538	<i>E. coli</i> ATCC 8739	<i>P. gingivalis</i> ATCC 33277	<i>P. acnes</i> ATCC 14916	<i>C. albicans</i> ATCC 10231	<i>C. tropicalis</i> TISTER 5306
Lac 0.25%w/v	17.7 $\pm$ 0.6	18.3 $\pm$ 0.6	24.3 $\pm$ 0.6	24.0 $\pm$ 1.7	13.3 $\pm$ 2.1	10.3 $\pm$ 0.6
Lac 1%w/v	16.7 $\pm$ 1.5	19.3 $\pm$ 3.2	24.0 $\pm$ 1.0	24.0 $\pm$ 1.7	14.7 $\pm$ 1.5	10.7 $\pm$ 0.6
Lac 2%w/v	16.7 $\pm$ 1.5	19.0 $\pm$ 3.5	23.3 $\pm$ 0.6	23.7 $\pm$ 0.6	15.7 $\pm$ 1.5	11.7 $\pm$ 0.6
Lac 4%w/v	16.3 $\pm$ 1.5	21.0 $\pm$ 0.0	24.0 $\pm$ 1.0	23.3 $\pm$ 1.2	17.3 $\pm$ 0.6	14.0 $\pm$ 1.0
DC 0.25%w/v	15.3 $\pm$ 0.6	15.3 $\pm$ 0.6	22.3 $\pm$ 0.6	41.0 $\pm$ 1.0	17.7 $\pm$ 1.2	10.7 $\pm$ 1.2
DC 1%w/v	17.3 $\pm$ 0.6	17.7 $\pm$ 0.6	23.3 $\pm$ 0.6	43.3 $\pm$ 1.2	18.3 $\pm$ 0.6	10.7 $\pm$ 1.2
DC 2%w/v	17.3 $\pm$ 0.6	18.0 $\pm$ 0.0	22.7 $\pm$ 0.6	43.0 $\pm$ 2.7	19.3 $\pm$ 1.2	10.3 $\pm$ 0.6
DC 4%w/v	15.7 $\pm$ 1.2	16.3 $\pm$ 0.6	21.3 $\pm$ 0.6	42.0 $\pm$ 1.0	15.3 $\pm$ 1.2	0.0 $\pm$ 0.0
PEG 400	13.0 $\pm$ 0.0	12.0 $\pm$ 0.0	21.3 $\pm$ 0.6	22.3 $\pm$ 1.2	13.3 $\pm$ 0.6	9.7 $\pm$ 0.6
GS 20 μ g/disc	30.8 $\pm$ 0.4	25.3 $\pm$ 0.2	28.6 $\pm$ 0.4	41.2 $\pm$ 1.3		
Fu 50 μ g/disc					25.5 $\pm$ 0.1	28.1 $\pm$ 0.7

Lac = Lac dye, DC = D&C red no.27, PEG = polyethylene glycol (a solvent for dissolved the dyes), GS = gentamicin sulfate, and Fc = fluconazole.

Antimicrobial activity

The antimicrobial activity results are presented in Table 2. Regarding antibacterial activity, all tested concentrations of lac dye and D&C red No. 27 effectively inhibited *S. aureus* and *E. coli*, respectively. Lac dye exhibited a stronger inhibitory effect against *P. gingivalis*, a pathogen responsible for periodontal disease, compared to D&C red No. 27. Specifically, lac dye at concentrations of 0.25% w/v and higher was able to inhibit *P. gingivalis*, while D&C red No. 27 required concentrations of 1% and 2% w/v to achieve inhibition. In comparison, lac dye exhibited only minimal inhibition against *P. acnes*, the bacteria responsible for acne, whereas D&C red No. 27 displayed the most potent inhibitory effect against *P. acnes* among all the microorganisms tested. This was evident as the inhibition zone for all concentrations of D&C red No. 27 was twice as large as those of lac dye and PEG400. Regarding antifungal activity, both lac dye and D&C red No. 27 were found to be less effective in inhibiting *C. albicans* and *C. tropicalis* when compared to PEG400. This was evident from the small inhibition zones observed for both dyes in comparison to PEG400, indicating a low antifungal efficacy. Only a few studies aligned with the results of this research, showing that lac dye had antibacterial effects against *S. aureus* and *E. coli* (Dey and Ghosh, 2018; Do *et al.*, 2023). Additionally, it was found that altering the chemical structure of lac dye, specifically through methylation of the carboxylic group in one of its key components, increased its efficacy against fungi responsible for plant diseases. These fungi included species such as *Alternaria solani*, *Curvularia lunata*, *Helminthosporium oryzae*, *Verticillium* sp., an *Erysiphe pisi*

(Srivastava *et al.*, 2017). However, no research has yet shown that lac dye has inhibitory effects on *P. gingivalis*, *P. acnes*, *C. albicans*, or *C. tropicalis*. This study is the first to investigate these microorganisms. Currently, no information is available on the antimicrobial activity of D&C red No. 27. However, there is existing data on the antimicrobial activity of D&C red No. 28, which belongs to the same xanthene dye group but differs in chemical structure, as shown in Figure 2. D&C red No. 28 demonstrated high inhibitory activity, with inhibition zones ranging from 15 to more than 20 mm, against *Bacillus cereus*, *E. coli*, *Salmonella indica*, and *S. aureus*. This activity was observed at concentrations starting from 16 mg/ml and extending up to 200 mg/ml (Selim *et al.*, 2012). Moreover, D&C red No. 28 was also capable of inhibiting methicillin-resistant *Staphylococcus aureus* (MRSA), with a minimum inhibitory concentration (MIC) of 25 μ g/ml (Rasooly and Weisz, 2002). Thus, this study marks the first reported instance of D&C red No. 27 exhibiting antimicrobial effects.

Lac dye is approved as a food additive in countries such as China, Japan, and Korea (Sharma *et al.*, 2020). In India, the Acceptable Daily Intake (ADI) is established at 2 mg/kg/day (Francis, 1996), whereas Japan sets the permissible usage levels in food between 5 to 100 mg/kg of foods, depending on the product (Delgado-Vargas and Paredes-López, 2003). Beyond its application in food, lac dye is also employed in cosmetic products for skin use, as well as for dyeing wool and silk fabrics (Sharma *et al.*, 2020). The safety evaluation of lac dye, based on single-dose and 90-day repeated oral toxicity studies, revealed no substance-related toxicity in Sprague-Dawley rats. A single dose of

lac dye at 5000 mg/kg body weight did not result in any fatalities, indicating an oral LD₅₀ of > 5000 mg/kg body weight. In the 90-day study, transient salivation was noted in the 200 and 500 mg/kg body weight groups, though there were no associated histopathological changes in the salivary glands. Additionally, non-adverse effects such as red-purple pigmentation on the surface of the femur and skull were observed in the 500 mg/kg group. No adverse effects were identified across all parameters examined, establishing 500 mg/kg body weight as the no-observed-adverse-effect level (NOAEL). Furthermore, genotoxicity studies indicated that lac dye was neither mutagenic nor clastogenic (Park *et al.*, 2022).

D&C red No. 27 is approved by US FDA for use as a color additive in general drugs and cosmetics. However, this color is not allowed for use in areas such as the eyes, in injections, or in surgical sutures (U.S. Food and Drug Administration, 2022). Following the evaluation of chronic toxicity studies in Charles River Sprague-Dawley CD rats and CD-1 mice, which were exposed to a lifetime dietary concentration of 2.0% and 1.0%, respectively, D&C red No. 27 was confirmed to be safe for use in drugs and cosmetics. The maximum acceptable daily intake has been established at 1.25 mg/kg body weight per day (National Archives and Records Administration, 1982).

Based on the safety data for the two dyes mentioned above, their use in appropriate quantities is expected to be safe for consumers.

Conclusions

Besides their safe application as colorants in food, pharmaceuticals, and cosmetics for aesthetic enhancement, lac dye and D&C red No. 27 also displayed biological activity. Lac dye demonstrated antioxidant activity that was four times greater than ascorbic acid and 200 times greater than D&C red No. 27. D&C red No. 27 showed anti-inflammatory activity approximately six times more effective than diclofenac sodium, whereas lac dye caused inflammation in the thermal inhibition of egg albumin protein denaturation assay. Despite this, lac dye still exhibited anti-inflammatory effects through other mechanisms. Both dyes effectively inhibited *S. aureus*, *E. coli*, and *P. gingivalis*. D&C red No. 27 had the strongest inhibitory effect on *P. acnes*, while lac dye did not inhibit against this bacterium. Neither dye was effective in inhibiting *C. albicans* and *C. tropicalis*. Based on this biological data, it is clear that dyes may offer health benefits when used appropriately and could be

valuable in the formulation of foods, drugs, and cosmetics in the future.

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