Research Article

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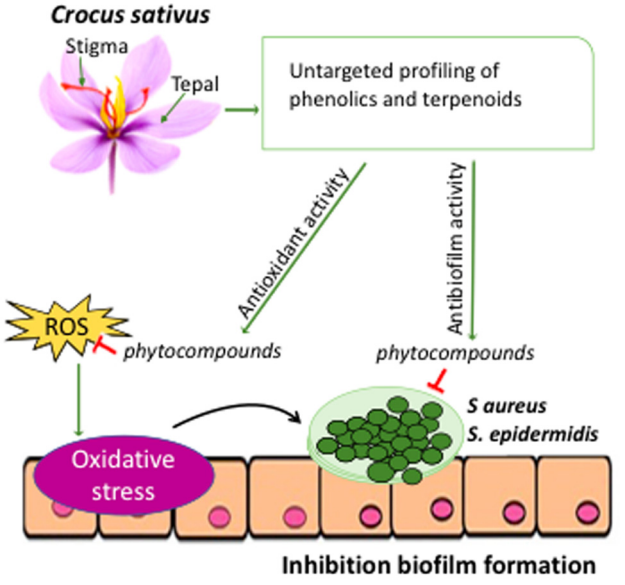
Phytochemical profiling, antibacterial and antioxidant properties of *Crocus sativus* flower: A comparison between tepals and stigmas

https://doi.org/10.1515/chem-2022-0155
received February 18, 2022; accepted April 6, 2022

Abstract: Several studies have demonstrated that stigmas of *Crocus sativus* contain several bioactive compounds with potential health-promoting properties. However, during the processing of stigmas, large amounts of floral bio-residues are normally discarded as by-products. In this study, using untargeted metabolomics, the comprehensive phytochemical composition of *C. sativus* stigma and tepals was investigated. Moreover, the antibacterial and anti-biofilm properties of the extracts of *C. sativus* stigmas and tepals were compared. The study was carried out using two methicillin-resistant staphylococcal reference strains (i.e., *Staphylococcus aureus* ATCC 43300 and Staphylococcus epidermidis ATCC 35984), representing important Gram-positive biofilm-forming human pathogens. The antibacterial properties were correlated with total polyphenol content, total terpenoid content, and *in vitro* antioxidant properties of tepals and stigmas. The results demonstrated that stigma and tepal extracts, at the sub-toxic concentrations, were able to interfere with biofilm formation by ATCC 43300 and ATCC 35984. Besides, the higher antibacterial activity of tepals than stigmas was associated with higher levels of phycompounds. Therefore, our results demonstrated that *C. sativus* stigmas and bio-residues, such as tepals, are potential antioxidant sources and good candidates as antibacterial agents to prevent biofilm formation. Taken together, these findings showed that *C. sativus* could be used as functional ingredient by the food and pharmaceutical industries.

Keywords: antibacterial activity, anti-biofilm, metabolomics, bio-residues, polyphenols, saffron



Graphical abstract

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1 Introduction

Saffron, the dried stigmas of the flowers of *Crocus sativus* L. Iridaceae (*C. sativus*), is considered the most valuable part of the flower. However, stigmas represent only 7.4% of *C. sativus* flowers. Therefore, large amounts of floral bio-residues, such as tepals, are generated and wasted in the production of saffron. The detailed chemical composition and health-promoting properties of saffron, such as antioxidant, hypolipidemic, antihypertensive, immunomodulatory, antimicrobial, antitumor, and antidepressant, have been characterized and recently reviewed [1–3]. Among the bioactive molecules, the antioxidant properties of crocin, picrocrocin, safranal have been previously demonstrated [4]. The beneficial properties of saffron have also been attributed to other terpenes and polyphenolic compounds [2,5]. Recent studies have reported that other plant parts also, such as *C. sativus* tepals, are rich in bioactive molecules, mostly polyphenols [6–11], and tepal extracts showed several biological activities, including antioxidant activity [6,12–16] and anti-diabetic properties [9,17].

Recently, increasing attention is devoted to antimicrobial properties exerted by phytonutrients, such as polyphenols and carotenoids. The effects exerted by phytonutrients on different factors which are critical for bacterial pathogenicity are being explored as a novel approach to combat bacterial pathogens [18–21]. Antimicrobial properties of crocin and safranal, two important bioactive components in *C. sativus*, against *Helicobacter pylori* [22] and *Candida* spp. [23] have been described and safranal showed a higher biological activity compared to crocin. Antimicrobial effects against several bacterial strains including *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella Dublin* [17,24–26] are also exerted by *C. sativus* petal and stamen extracts. Despite their relevance, these studies are incomplete, as bacteria must adhere and even form biofilms to colonize and thrive in most matrixes such as foodstuffs, medical devices, or even the skin. Moreover, previous papers reported that bacterial biofilms, refractory to antibiotic treatment and host immune systems, are involved in most chronic infections [27] and anti-biofilm agents are being searched for urgently.

Therefore, in this work, to increase the overall profitability of *C. sativus*, polyphenol and terpenoid contents and profiles, together with *in vitro* antioxidant activities and antibacterial and anti-biofilm properties of *C. sativus* tepal and stigma extracts, were compared. The antibacterial activities of saffron, tepal and stigma extracts were studied using two methicillin-resistant staphylococcal reference strains (*Staphylococcus aureus* ATCC 43300 and *Staphylococcus epidermidis* ATCC 35984). These strains are gram-positive biofilm-forming human pathogens that can cause human diseases and community-acquired infections, increasing morbidity and mortality [27,28].

2 Materials and methods

2.1 Reagents

Reagents for cell culture were obtained from Euroclone (Euroclone, Italy). All chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was supplied by Invitrogen (Invitrogen, Carlsbad, CA, USA). Human Dermal Fibroblast was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA).

2.2 Plant material

Crocus sativus L. Iridaceae was kindly supplied by a local farm. Briefly, the flowers were carefully handled to recover the stigmas and tepals. After, collection, stigmas and tepals were frozen and put in the freeze-drier (Heto Dry Winner 685, Denmark). The freeze-dried samples were weighed, ground and then vacuum-packed and stored at room temperature in the dark.

2.3 Extraction procedure for high-resolution-mass spectrometry (HRMS) analyses

The dried samples of *C. sativus* tepals and stigmas were extracted (using a material to solvent ratio of 1:10 w/v) with a modified version of the method previously described by Matyash et al. (2008) [29]. Briefly, the samples were dissolved in a mixture of 80% aqueous methanol and methyl-*tert*-butyl ether (MTBE) (1:1 v/v), mixed by vortexing for 2 min, and then ultrasonic processed for 5 min. Then, upon 10 min of incubation at room temperature, the samples were centrifuged at 6,000×*g* for 10 min. The upper (organic) phase (300 µL) was collected in a 1.5 mL microcentrifuge tube and dried. The extracted lipids were then dissolved in 300 µL of isopropanol/methanol/water (60:35:5, v/v) and collected in amber vials for untargeted lipidomic analysis. Regarding the hydrophilic fraction, an aliquot of 500 µL of

the lower phase (i.e., the aqueous methanol fraction) was collected and stored in amber vials until the further untargeted phenolic-profiling analysis.

2.4 Untargeted profiling of phenolics and terpenoids

2.4.1 Ultra-high-pressure liquid chromatography-quadrupole time of flight (UHPLC-QTOF) analysis of polyphenols

A metabolomics-based approach was used to comprehensively annotate phenolic compounds in the dried samples, using the instrumental conditions reported previously by Rocchetti et al. [30]. The compounds' separation was achieved using an Agilent Zorbax Eclipse Plus C18 column (50 mm × 2.1 mm, 1.8 µm), with water-acetonitrile linear gradient elution from 6 to 94% of acetonitrile in 32 min. Samples were analyzed under positive ionization mode (ESI+), using a full-scan mode with an *m/z* range of 100–1200 (scan rate: 0.8 spectra/s and mass resolution 30,000 FWHM). The injection volume was 6 µL and the sequence was randomized, injecting pooled quality control (QC) samples throughout the sequence. In this regard, QC samples were analyzed using 12 precursors per cycle, considering an *m/z* range of 50–1,200 *m/z* and setting typical collision energies of 10, 20, and 40 eV. After that, Agilent Profinder B.07 software (Agilent Technologies) was used to process raw spectral data, according to the 'find-by-formula' algorithm. The highest degree of confidence in the putative annotation (level 2) was achieved using monoisotopic mass information together with the isotopic pattern and using a 5-ppm tolerance for mass accuracy. The find-by-formula algorithm was used against the comprehensive database Phenol-Explorer 3.6. The post-acquisition data filtering, baselining, and normalization of MS features were done using the software Mass Profiler Professional (version: B.12.06, from Agilent Technologies), as reported previously [30]. Finally, the cumulative normalized abundance values of the different phenolic classes were converted into semi-quantitative data, using standard solutions of pure compounds representative of their phenolic class [30]. In this regard, the following standard compounds were used: cyanidin (for anthocyanins), quercetin (for flavonols), catechin (for flavan-3-ols), luteolin (for other remaining flavonoids), sesamin (for lignans), resveratrol (for stilbenes), ferulic acids (for phenolic acids), and tyrosol (for other remaining phenolics). The results were expressed as mg equivalents (Eq.)/g dry matter (DM).

2.4.2 Untargeted UHPLC-Orbitrap analysis of terpenoids

The identification of terpenoids in the *C. sativus* tepals and stigmas was made using a UHPLC-MS lipidomics-based approach, based on a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish UHPLC pump and equipped with a HESI-II probe (Thermo Scientific, USA). The chromatographic separation was done through a BEH C18 column (2.1 mm × 100 mm, 1.7 µm) maintained at 40°C. The mobile phase consisted of: (A) 5 mM ammonium formate and 0.1% formic acid in water/methanol (95/5, v/v), and (B) 5 mM ammonium formate and 0.1% formic acid in 2-propanol/methanol/water (65/30/5, v/v/v). The linear gradient and flow rate increased from 10% to 100% in 30 min. For the full-scan MS analysis, the acquisition was made in both positive and negative ionization modes, at a nominal resolution of 70,000 FWHM. The automatic gain control (AGC) target and maximum injection time (IT) were 1×10^6 and 100 ms, respectively. Also, the injection sequence was randomized and pooled QC samples were injected using a data-dependent (Top $N = 3$) MS/MS mode (full scan mass resolution reduced to 17,500 at *m/z* 200, AGC value of 1×10^5, maximum IT of 100 ms, and isolation window of 1.0 *m/z*). The collected UHPLC-HRMS data (as .RAW file) were then converted into .abf file using the Reifycs Abf converter and then further processed using the software MS-Dial (version 4.38) and MS-Finder [31]. The annotation via spectral matching in MS-Dial was done using the in-house database LipidBlast, excluding the retention time from calculating the total identification score. In this regard, the identification process was based on mass accuracy, isotopic pattern, and spectral matching. In addition, the software MS-Finder provided the *in-silico* annotation of the not fully annotated MS/MS features, considering the compounds reported on Lipid Maps and Food Database libraries. Finally, to provide a cumulative semi-quantitative content of the terpenoids annotated, a standard solution of beta-carotene was analyzed under the same conditions of the samples. The results were expressed as mg equivalents (Eq.)/g dry matter (DM).

2.5 *In vitro* antioxidant activity and phenolic contents

2.5.1 Determination of total polyphenol content and flavonoid content

Total polyphenolic content (TPC) in extract was evaluated following the Folin–Ciocalteu assay [32]. Briefly,

20 µL of extract was used. 0.1 mL of Folin–Ciocalteu phenol reagent and 0.3 mL sodium carbonate solution (20%) were added to the tubes. After incubation for 40 min at 37°C, absorbance was evaluated at 765 nm using a UV-Vis spectrophotometer (UV Analyst-CT 8200). Blank reagents were prepared using 20 µL of water and treated as described above. Gallic acid (GA) was used to develop a 0.1–1.3 mg/mL standard curve. The experiments were carried out in triplicate and TPC were expressed as milligrams of GA equivalent per g of dry weight (mg GAE/g DW).

Total flavonoid content (TFC) was quantified according to the method of Kim et al. [33]. Briefly, 500 µL of extracts were used. 150 µL of $NaNO_2$ (5%) were added to the tubes. At the end of incubation (5 min) at room temperature, 150 µL of 10% $AlCl_3$ were added and samples were further incubated for 1 min at room temperature. After that, 2 mL of 1 M NaOH were added. After incubation for 15 min, absorbance was evaluated at 510 nm against the blank using a UV-Vis spectrophotometer (UV Analyst-CT 8200). Catechin was used as a standard for the calibration curve (0–150 µg/mL). All the experiments were done in triplicate and results were expressed as milligrams of catechin equivalent (CE) of g of dry weight (mg CE/g DW).

2.5.2 Oxygen radical absorbance capacity (ORAC) assay

The total antioxidant capacity of *C. sativus* tepal and stigma extracts was evaluated using the ORAC method. ORAC assay was carried out on a plate reader [34]. Briefly, 25 µL of the diluted sample, blank, or trolox calibration solutions were mixed with 150 µL of 0.08 µM fluorescent probe, fluorescein, and incubated for 15 min at 37°C. Subsequently, 25 µL of 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) solution (150 mM) was added as a peroxyl radical generator. The fluorescence was measured every 2 min for 4 h, using fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All samples were analyzed in triplicate. The final ORAC values were calculated using the net area under the decay curves and were expressed as millimoles of Trolox equivalents (TE)/g of sample.

2.5.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Radical scavenging activity of *C. sativus* tepal and stigma extracts was evaluated by monitoring the decrease in absorbance at 517 nm of methanolic solution of the radical DPPH (final concentration 90 µM) incubated for 40 min in the absence (Ab) or in the presence (Ac) of the samples [35].

Radical scavenging activity was calculated by the following formula:

$$\% \text{ inhibition} = [(Ab - Ac)/Ab] \times 100.$$

Polyphenolic concentration (expressed as µg GAE/mL) in the extracts necessary to decrease the initial concentration of DPPH by 50% (IC_{50}), under the experimental condition was calculated [35].

2.6 Cell culture and incubation in the absence or presence of extracts

Human dermal fibroblasts (HuDE) cell lines were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% of glutamine, 1% of penicillin/streptomycin, and 1% of essential amino acids at 37°C and 5% CO_2. HuDE cells were seeded at a density of 5×10^3 into a 96-well plate and incubated at 37°C in an atmosphere of 5% CO_2. After plating overnight, 100 µL of extracts of *C. sativus* tepals or stigmas containing increasing concentrations of polyphenols (0–350 µg GAE/mL) were added to each well. The cells were then incubated at 37°C for 48 h. Cell viability was evaluated using the methyl thiazolyl tetrazolium (MTT) assay [36].

ROS formation in HuDe cells treated with extracts of *C. sativus* tepals and stigmas was evaluated using H_2DCFDA. Cells were seeded 24 h before treatment (25×10^3 cells/well). At the end of incubation, cells were washed twice with in cold phosphate-buffered saline (PBS) and then incubated for 45 min with pre-warmed PBS containing the probe (final concentration of 10 µM). After incubation in the dark at 37°C, cells were washed twice in PBS. The fluorescence of the labelled cells was measured in a Synergy microplate reader using an excitation wavelength of 488 nm and emission wavelength of 530 nm [37,38].

2.7 Antibacterial activity

2.7.1 Strains and growth media

Two methicillin-resistant and high biofilm producer reference strains, *Staphylococcus aureus* ATCC 43300 (clinical isolate) and *Staphylococcus epidermidis* ATCC 35984 (isolated from a patient with device-associated sepsis) [39], were used throughout the study. The methicillin-susceptible *S. aureus ATCC* 29213 was also included to verify the accuracy of the microdilution test procedure.

Brain heart infusion (BHI) agar, Müller-Hinton cation-adjusted broth (CAMHB), Müller-Hinton agar (MHA), and Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) were used in the study. Isolates were maintained in glycerol at −70°C and subcultured twice on BHI agar before testing.

2.7.2 Susceptibility tests

The Minimum Inhibitory Concentration (MIC), i.e., the lowest concentration of *C. sativus* tepal and stigma extracts able to inhibit visible bacterial growth after incubation, was determined in CAMHB by the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [40].

2.7.3 Biofilm formation assays

The ability of the extracts to inhibit biofilm formation was evaluated as described previously [41]. Briefly, bacteria were grown overnight in TSB containing 1% glucose at 37°C. Overnight bacterial suspensions were prepared to yield final inocula of ~2×10^8 colony-forming units (CFU)/mL; then 100 µL of bacterial culture and 100 µL of the extract were added to each well of a 96-well microplate. Wells containing 100 µL of the bacterial suspension and 100 µL of TSB without the extract were the positive controls. After 24 h of incubation, wells were washed 3 times in PBS, dried for 1 h at 60°C, and stained with Hucker's crystal violet. After 3 washes in sterile water, wells were inoculated with 100 µL of 95% EtOH and shaken for 10 min. Biofilm formation was quantified by measuring absorbance at 690 nm with a Multiskan Ascent apparatus (Thermo Scientific, Waltham, MA, USA).

2.7.4 Scanning Electron Microscopy (SEM) analysis of biofilm

For morphological studies, samples of biofilms grown overnight at 37°C on glass slides were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C and washed with the same buffer supplemented with 7% sucrose, postfixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer for 2 h at 4°C, dehydrated with EtOH and critical point dried. Samples were mounted on aluminum stubs by graphite glue and coated with a thin (20 Å) gold film using an EMITECH K550 (Ashford, England) sputtering device. Observations were performed with a Zeiss Supra 40 apparatus.

2.8 Statistical analysis

Each experiment was performed at least in triplicate. All data are presented as mean value ± SD. Significant differences between groups were calculated with the paired Student's *t*-test by using GraphPad software. *p*-values lower than 0.05 were considered statistically significant. The MS data related to polyphenols and terpenoids were then elaborated through the software Agilent Mass Profiler Professional B.12.06 [30]. In this regard, a combined dataset was exported into SIMCA 13 (Umetrics, Malmo, Sweden), Pareto scaled and elaborated for orthogonal projections to latent structures discriminant analysis (OPLS-DA). The discriminant compounds between stigma and tepal extracts were then evaluated through the variable importance in projection approach (VIP), considering those compounds characterized by a VIP discriminant score >1.2. Also, for each VIP marker compound, the Log Fold-Change (FC) variations were calculated. Finally, Pearson's correlation coefficients between the MS-data and the different biological assays were calculated using the software IBM PASW Statistics 26.0 (SPSS Inc.).

3 Results

3.1 Phenolic profiling of the tepal and stigma extracts

The UHPLC-QTOF analysis of tepal and stigma extracts revealed a diverse phenolic composition. In this regard,

Table 1: Semi-quantitative results considering the different phenolic subclass and the total terpenoid content in tepal and stigma extracts. The results are expressed as mean value ± standard deviation ($n = 3$)

Class	Stigmas (mg Eq./g DM)	Tepals (mg Eq./g DM)
Anthocyanins	4.6 ± 0.8	34.6 ± 3.5**
Flavonols	0.3 ± 0.1	2.0 ± 0.0**
Flavan-3-ols	0.1 ± 0.0	0.4 ± 0.2
Other flavonoids	0.6 ± 0.1	2.3 ± 0.2**
Lignans	8.9 ± 2.9	1.8 ± 0.8*
Stilbenes	0.7 ± 0.1	0.1 ± 0.00
Phenolic acids	0.7 ± 0.1	0.9 ± 0.1
Other phenolics	9.8 ± 1.0	4.8 ± 0.4**
Terpenoids	0.6 ± 0.2	0.2 ± 0.1

**$p < 0.01$ vs stigma extracts; *$p < 0.05$ vs stigma extracts; DM = dry matter.

the untargeted phenolic profiling consisted of 452 compounds, namely 66 anthocyanins, 17 flavan-3-ols, 67 flavonols, 94 other flavonoids (including flavones, flavanones, dihydrochalcones, dihydroflavonols, chalcones, and isoflavonoids), 29 lignans, 79 low-molecular-weight phenolics (i.e., tyrosol equivalents), 92 phenolic acids (mainly hydroxybenzoic and hydroxycinnamic acids), and 8 stilbenes (Table S1). The semi-quantitative approach based on representative standard compounds per phenolic class allowed detecting marked differences between the different extracts. Overall, the total phenolic content differed between stigma and tepal extracts, being 25.22 and 46.92 mg/g, respectively (Table 1). Going into detail of each phenolic class, it was evident from Table 1 that tepals were very abundant in flavonoids compared to stigmas, with the anthocyanins presenting the highest cumulative content (i.e., 34.57 mg/g). On the other hand, stigmas showed higher cumulative values for lignans and low-molecular-weight phenolics (i.e., tyrosol equivalents), being 8.89 and 9.83 mg/g, respectively. Finally, no significant ($p > 0.05$) differences were observed between the two extracts when considering phenolic acids and stilbenes (Table 1). Regarding the significant differences observed in anthocyanins content, tepals were exclusively abundant in some compounds, such as cyanidin, delphinidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, pigment A (and its isomer peonidin 3-*O*-(6″-*p*-coumaroyl-glucoside)), petunidin 3,5-*O*-diglucoside, and malvidin 3,5-*O*-diglucoside (Table S1). A comprehensive list reporting all the compounds annotated in our experimental conditions can be found as Supplementary Material (Table S1), considering their relative abundance values and composite mass spectra (mass and abundances combinations).

3.2 Terpenoid profiling of the tepal and stigma extracts

In this work, we used a lipidomic-like approach to identify those terpenoids characterizing both tepal and stigma extracts. Overall, the UHPLC-Orbitrap-mass spectrometry approach allowed annotating 15 compounds, including typical saffron terpenoids, such as crocin, crocin 3, dicrocin, and tricrocin. Besides, other compounds (e.g., carotenoids and xanthophylls) such as beta-carotene, violaxanthin, and zeaxanthin have been detected (Table S1). After that, a standard of beta-carotene allowed us to check the semi-quantitative differences when considering both stigma and tepal extracts. As can be observed from Table 1, stigmas showed a total terpenoid content numerically higher than tepals, being 0.63 vs 0.24 mg/g, respectively. However, no significant differences ($p > 0.05$) have been detected according to the one-way ANOVA and Duncan post-hoc test. Regarding the distribution of each main compound detected, we found that crocin 3 (also known as beta-D-Gentiobiosyl crocetin) was very abundant in stigmas, showing a LogFC value of 10.36 compared with tepals. Similar trends were observed for the other typical carotenoids (such as crocin, dicrocin, and tricrocin; Supplementary Material).

3.3 Multivariate statistical discrimination of tepals and stigmas

To provide discrimination between tepals and stigmas, a supervised statistical approach based on multivariate OPLS-DA was used. Overall, the OPLS-DA score plot (Figure S1) provided clear discrimination on the orthogonal component between two different saffron extracts. The model was characterized by significant goodness of fitting and prediction parameters ($R^2X = 0.773$; $R^2Y = 1$; $Q^2 = 1$). Therefore, to extrapolate each plant part's best marker compounds, the variables' selection method VIP was used. The discriminant compounds are reported in Table 2, considering their VIP score (cut-off > 1.24), subclass, and LogFC value when comparing tepals to stigmas. Overall, the VIP selection method showed 38 discriminant compounds (excluding the isomeric forms typical of untargeted MS experiments). First of all, phenolic compounds were found to possess a wider weight in discriminating the two extracts, although the highest VIP score was detected for a terpenoid, namely, crocin 3 (VIP score = 1.24). Regarding polyphenols, we found an abundance of flavonoids as discriminant compounds (28 markers), followed by phenolic acids (6 markers), and 3 low-molecular-weight phenolics. Also, among the flavonoids, we found 3 compounds showing the highest LogFC value, namely, 3-*O*-glucosyl-rhamnosyl-glucosides of kaempferol and quercetin (LogFC = 22.58), followed by the prodelphinidin trimer GC-GC-C (LogFC = 21.48). Other marker compounds exclusively characterizing tepal extracts were 1-sinapoyl-2,2′-diferuloylgentiobiose (VIP score = 1.24; LogFC = 20.88), 2,5-di-*S*-glutathionyl caftaric acid (VIP score 1.24; LogFC = 19.27). Regarding stigma extracts, we found some significant markers such as cyanidin 3-*O*-galactoside (VIP score = 1.23; LogFC = −22.82), protocatechuic acid (and its isomer 3,5-dihydroxybenzoic acid) and gallic acid 3-*O*-gallate (averaged LogFC variations of −20.4).

Table 2: VIP marker compounds better discriminating the comparison "tepals vs stigmas." The compounds are reported together with their class, VIP score (from OPLS-DA; cut-off > 1.24), and LogFC values

Class	Discriminant compound	VIP score (OPLS-DA)	LogFC values [tepals vs stigmas]
Terpenoids	Crocin 3 (beta-D-gentiobiosyl crocetin)	1.24 ± 0.03	−10.36
Flavonoids	Isorhamnetin 7-*O*-rhamnoside/isorhamnetin 3-*O*-glucoside	1.24 ± 0.03	6.08
	Kaempferol 3-*O*-glucosyl-rhamnosyl-glucoside/quercetin 3-*O*-rhamnosyl-rhamnosyl-glucoside	1.24 ± 0.03	22.58
	Prodelphinidin trimer GC-GC-C	1.24 ± 0.02	21.48
Phenolic acids	1-Sinapoyl-2,2′-diferuloylgentiobiose	1.24 ± 0.02	20.88
Flavonoids	Malvidin 3,5-*O*-diglucoside	1.24 ± 0.04	5.94
	Scutellarein/luteolin/kaempferol	1.24 ± 0.03	5.24
	Kaempferol 3-*O*-acetyl-glucoside/quercetin 3-*O*-acetyl-rhamnoside	1.24 ± 0.04	6.72
	Chrysoeriol 7-*O*-(6″-malonyl-apiosyl-glucoside)	1.24 ± 0.05	4.94
	Dihydromyricetin 3-*O*-rhamnoside	1.24 ± 0.04	5.57
	(−)-Epigallocatechin/(+)-gallocatechin	1.24 ± 0.03	−2.17
Phenolic acids	2,5-di-*S*-Glutathionyl caftaric acid	1.24 ± 0.02	19.27
	p-Coumaroyl malic acid	1.24 ± 0.03	3.76
Flavonoids	Kaempferol 3-*O*-(2″-rhamnosyl-6″-acetyl-galactoside) 7-*O*-rhamnoside	1.24 ± 0.02	18.84
	Delphinidin 3-*O*-glucoside	1.24 ± 0.05	6.42
	Malvidin 3-*O*-(6″-*p*-coumaroyl-glucoside)	1.24 ± 0.04	4.65
	Petunidin 3,5-*O*-diglucoside	1.24 ± 0.06	5.23
	Kaempferol 7-*O*-glucoside	1.24 ± 0.03	−1.36
	Phloridzin	1.24 ± 0.05	2.55
	Malvidin 3-*O*-glucoside	1.24 ± 0.05	5.68
	Delphinidin 3-*O*-sambubioside	1.24 ± 0.06	4.89
	Apigenin 7-*O*-(6″-malonyl-apiosyl-glucoside)	1.24 ± 0.05	2.20
	Theaflavin	1.24 ± 0.04	2.52
Phenolic terpenes	Carnosol	1.24 ± 0.05	−2.79
Other polyphenols	*p*-HPEA-EDA	1.24 ± 0.07	−2.56
Flavonoids	Luteolin 7-*O*-(2-apiosyl-glucoside)/kaempferol 3-*O*-xylosyl-glucoside	1.24 ± 0.06	3.74
Phenolic acids	Protocatechuic acid/3,5-dihydroxybenzoic acid	1.24 ± 0.07	−20.07
Flavonoids	Cyanidin 3-*O*-glucosyl-rutinoside	1.24 ± 0.07	2.17
	Kaempferide	1.24 ± 0.06	2.19
	Phloretin 2′-*O*-xylosyl-glucoside	1.24 ± 0.11	7.11
	Kaempferol 3-*O*-sophoroside/kaempferol 3,7-*O*-diglucoside/quercetin 3-*O*-galactoside 7-*O*-rhamnoside/quercetin 3-*O*-rhamnosyl-galactoside/quercetin 3-*O*-rutinoside	1.24 ± 0.09	0.95
	6″-*O*-Malonyldaidzin	1.24 ± 0.07	2.86
	Theaflavin 3-*O*-gallate/theaflavin 3′-*O*-gallate	1.24 ± 0.11	18.41
Other polyphenols	Esculetin	1.24 ± 0.11	2.24
Flavonoids	6-Geranylnaringenin	1.24 ± 0.08	−1.04
Phenolic acids	Caffeic acid 4-*O*-glucoside/caffeoyl glucose	1.24 ± 0.08	−4.35
	24-Methylcholestanol ferulate	1.24 ± 0.09	1.79

3.4 *In vitro* antioxidant activity and Pearson's correlations

The evaluation of TPC (as assessed by Folin–Ciocalteu assay) in the extracts confirm that the content differed between stigmas and tepals being 20 ± 3 and 36 ± 3 mg GAE/g, respectively ($p < 0.001$) (Table 3). TFC (as assessed spectrophotometrically) were also higher in tepals (5.6 ± 0.2 mg CE/g) compared to stigmas (2.4 ± 0.2 mg CE/g) ($p < 0.001$) (Table 3).

The *in vitro* antioxidant activity of tepals and stigmas was determined according to different methods in the water extracts, namely DPPH assay and ORAC assay (Table 3). DPPH scavenging ability of extracts was concentration-dependent and in Table 3, as shown from IC_{50} values. A lower IC_{50} value indicates a higher antioxidant activity, therefore tepal extracts showed a DPPH free radical scavenging activity higher compared to stigma extracts. The ORAC assay also confirmed these results; in this regard, the ORAC values detected in tepal extracts

Table 3: Total polyphenols, total flavonoids, and antioxidant activities of tepals and stigma extracts. The results are expressed as mean value ± standard deviation ($n = 4$)

	Stigma extracts	Tepal extracts
TCP (mg GAE/g)	20 ± 3	36 ± 3*
TFC (mg CE/g)	2.4 ± 0.2	5.6 ± 0.2*
ORAC value (mmol TE/g)	0.8 ± 0.1	1.7 ± 0.1*
DPPH-scavenging assay (IC_{50}) (μg GAE/mL)	28 ± 3	11 ± 2*

*$p \leq 0.001$ vs stigma extracts.

were 2-fold higher when compared with those observed in stigma extracts (Table 3).

Finally, Pearson's correlation coefficients were used to evaluate the contribution of TPC and TFC to the *in vitro* antioxidant activity values recorded in extracts. Overall, as can be observed in Supplementary Material, we found significant ($p < 0.01$) and positive correlation coefficients for TPC and TFC with the ORAC values, being 0.926 and 0.969, respectively. On the other hand, TPC and TFC values were significantly and negatively correlated to the DPPH values, recording coefficients of −0.907 ($p < 0.05$) and −0.943 ($p < 0.01$), respectively.

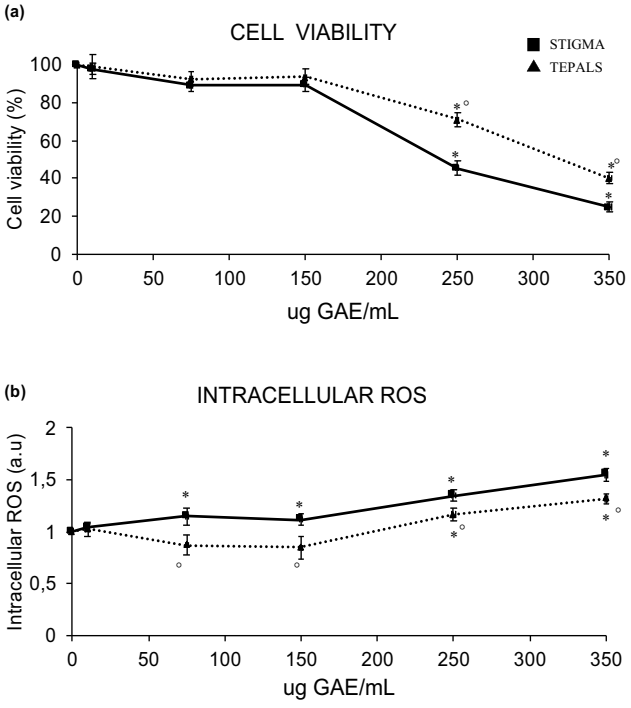

Figure 1: Cytotoxicity of tepal and stigma extracts. Effect of increasing concentration of *C. sativus* stigma (■) and tepal (▲) extracts on cell viability (a) and intracellular ROS formation (b) in human dermal fibroblasts. The cells were treated with increasing concentrations of extracts (0–350 μg GAE/mL) for 48 h. The results are expressed as mean value ± standard deviation ($n = 4$). *$p \leq 0.001$ vs cells incubated in the absence of extracts. °$p \leq 0.05$ vs cells incubated with the stigma extract.

3.5 Cell cytotoxicity

The potential cytotoxic activity of extracts was determined using MTT assay in HuDe cell line exposed to extracts with increasing concentration of polyphenols (0–350 μg GAE/mL). As reported in Figure 1a, extracts have been shown to reduce cell viability in a dose-dependent manner. The extracts showed no cytotoxicity toward HuDE cells at concentrations lower than 250 μg GAE/mL (Figure 1a). At higher concentrations, both stigma and tepal extracts induced a decrease in cell viability and an increase in ROS formation (Figure 1b).

3.6 Antibacterial and anti-biofilm activity

The MICs of stigma and tepal extracts were >512 μg GAE/mL against both tested strains (*S. aureus* ATCC 43300 and *S. epidermidis* ATCC 35984). Both strains were tested for biofilm production in the presence of subinhibitory concentrations (64–128 μg GAE/mL) of saffron extracts. A significant decrease in biofilm production of *S. aureus* ATCC 43300 and *S. epidermidis* ATCC 35984 was observed in the presence of extracts of *C. sativus* stigmas at both tested concentrations (Figure 2a and b). A significant ($p \leq 0.05$) decrease in biofilm production was also detected using extracts obtained from *C. sativus* tepals. The percentage decrease of biofilm formation of *S. aureus* ATCC 43300 ranged from 33.9 to 58.0% in the presence of *C. sativus* tepals. The percentage decrease of biofilm formation in *S. epidermidis* ATCC 35984 ranged from 27.3 to 34.3% (Figure 2c and d).

To further assess the anti-biofilm activity of the two extracts, biofilms formed by *S. aureus* ATCC 43300 and S. epidermidis ATCC 35984 were examined by SEM. Morphological analysis performed by SEM investigations on biofilms formed by *S. aureus* ATCC 43300 and *S. epidermidis* ATCC 35984, clearly shows the anti-biofilm activity of the two extracts, as the staphylococcal biofilm formation of the two pathogens on glass surfaces appeared markedly inhibited (Figure 3). Biofilms grown in the presence of extracts always exhibited a different three-dimensional structure, apparently with fewer cell layers and with some detachment areas, compared to the densely packed controls. In

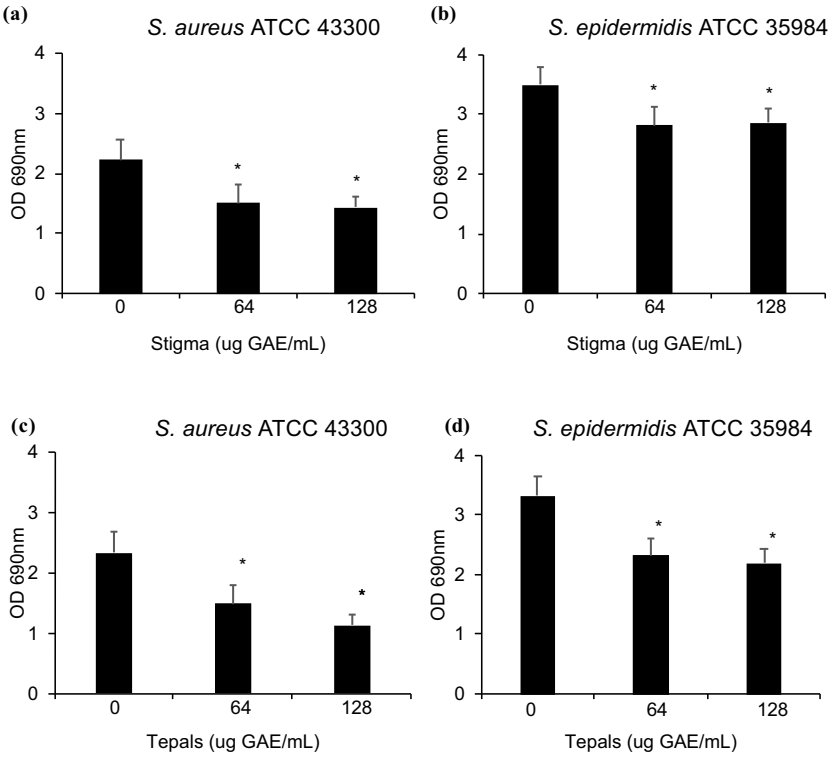

Figure 2: Antibiofilm activity of tepal and stigma extracts. *S. aureus* ATCC 43300 biofilm production in the absence and in the presence of sub-inhibitory concentration of extracts (a) *C. sativus* stigmas and (c) *C. sativus* tepals. *S. epidermidis* ATCC 35984 biofilm production in the absence and in the presence of sub-inhibitory concentrations of (b) *C. sativus* stigmas and (d) *C. sativus* tepals. Each value is mean OD 690 ± SD of four experiments. *$p \leq 0.05$ vs control. Polyphenol concentration in extracts is expressed as µg GAE/mL.

particular, in *S. aureus* ATCC 43300 bacterial culture, administration at sub-inhibitory concentrations (64 and 128 µg GAE/mL) of *C. sativus* stigmas extract (Figure 3a) and *C. sativus* tepals extract (Figure 3b) induced a clear reduction in the densely packed structure of biofilms with respect to the control biofilms. The loss of 3D structure can be referred as bacterial cell loss of contiguous cell grown, and growth of more dispersed bacteria.

A similar trend was observed as regards to *S. epidermidis* ATCC 35984 (Figure 3), where bacterial "towers" connected by channels is particularly evident in control biofilms, and disruption of biofilm grown was clearly detected after administration of extracts at the same conditions. In these cases, a higher influence was exerted by 128 µg/mL stigma extract (Figure 3c) with respect to the tepal one (Figure 3d).

4 Discussion

In this work, the bioactive properties of saffron have been demonstrated, and the roles of chemical components, including polyphenols and terpenoids, in stigmas have been characterized. Bioactive compounds of *C. sativus* and their semi-synthetic derivatives have been previously described as promising anti-Helicobacter pylori, antimalarial, and anti-leishmanial agents [2,3,5,42]. However, the chemical composition and bioactive properties of tepals have been less investigated.

Our findings demonstrated differences in the chemical composition of stigmas when compared to tepals. In this regard, the study of terpenoid profiling of tepal and stigma extracts showed a total terpenoid content higher than tepals. Furthermore, the UHPLC-QTOF analysis of tepal and stigma extracts showed a different polyphenolic content and composition. In particular, tepals showed higher TPC (in particular flavonoids) compared to stigmas, and anthocyanins levels were higher compared to stigma. On the other hand, stigmas showed higher cumulative values for lignans and low-molecular-weight phenolics. These findings are consistent with previous studies on qualitative fingerprinting of tepals and stigmas obtained from samples cultivated in multiple areas, including Abruzzo (Italy) using UHPLC-QTOF-MS/MS metabolomic-based approach [10,11] or by exploiting other analytical approaches, such as HPLC-PDA [9], LC-ESI- IT MS/MS [6,7], and HPLC-DAD [8].

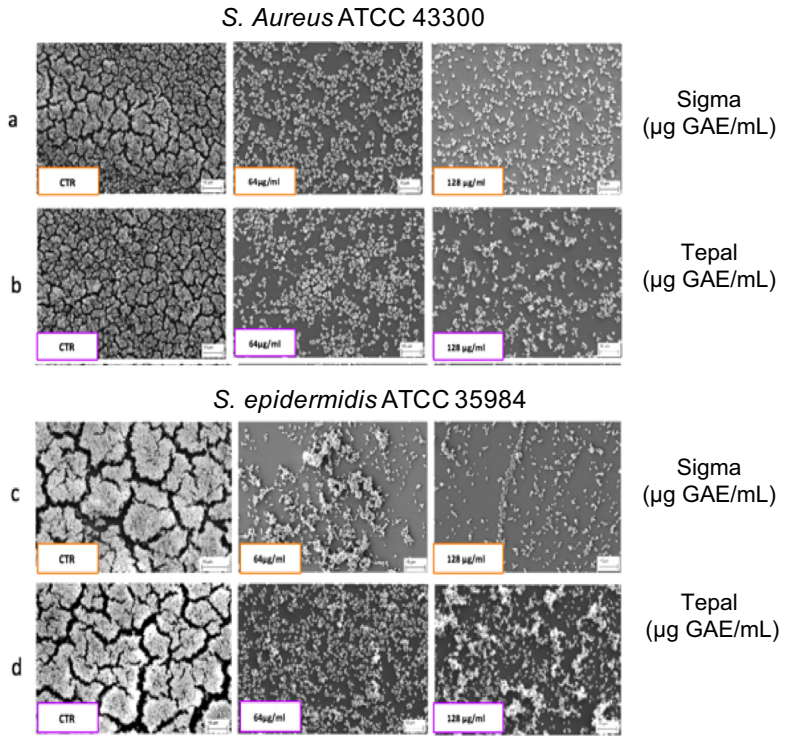

Figure 3: SEM images of biofilms production in the presence and absence of tepal and stigma extracts. SEM images of *S. aureus ATCC* 43300 biofilms on glass surfaces in the absence and presence of sub-inhibitory concentrations (64 and 128 µg GAE/mL) of (a) *C. sativus* stigmas extract and (b) *C. sativus* tepals extract. SEM images of *S. epidermidis* ATCC 35984 biofilms in the absence and in the presence of sub-inhibitory concentration (64 GAE and 128 µg GAE/mL) of (c) *C. sativus* stigmas extract and (d) *C. sativus* tepals extract. The selected images were chosen as the best representatives of the amount of biofilm on the glass surfaces. Polyphenol concentration in extracts is expressed as µg GAE/mL.

Besides using different spectrophotometric assays (DPPH and ORAC assays), our findings confirm that *C. sativus* tepal extracts exert a higher antioxidant activity than extracts obtained from stigmas, in agreement with previous studies [13,14]. These results could be related to higher levels of total phenols and flavonoids observed in the tepal extracts compared to stigma extracts. In fact, Pearson's correlation coefficients provided evidence that TFC and TPC were highly correlated to ORAC values. TPC and TFC in water extracts have been assessed by UV/VIS spectrophotometric methods. These methods are widely used and easy to measure the TPC in saffron and other plant products [9,13,43], even if it has been reported that interferences caused by non-phenolic substances, including proteins, amino acids, thiols, and vitamins, could impair their selectivity.

Extracts of *C. sativus* tepals and stigmas were then tested *in vitro* to evaluate their modulatory effects on cell viability and oxidative stress. We observed that *C. sativus* extracts at concentrations up to 250 µg GAE/mL did not exert a cytotoxic effect on HuDe cells. In contrast, cell viability was reduced at higher polyphenol concentrations with a significant increase in intracellular ROS levels. These results agree with previous studies that reported that polyphenols in cell culture media, at high levels, would have detrimental effects. Other authors using polyphenol-rich extracts from saffron [9,44] or different sources [37], as well as individual pure polyphenols [45], obtained similar results in different cell models. These data confirm opposite effects on intracellular ROS production induced by low or high doses of polyphenols [37,46].

The antibacterial activities of *C. sativus* tepal and stigma extracts against two methicillin-resistant and biofilm producer staphylococcal reference strains were evaluated at the subtoxic concentrations (0–128 µg GAE/mL). The antimicrobial properties of *C. sativus* extracts and some important bioactive components of saffron, namely crocin and safranal, have been previously investigated, showing biological properties toward *Helicobacter pylori, Candida* spp. [22,23], and of food-borne bacterial strains (*Bacillus cereus*, *Staphylococcus aureus, Salmonella enterica, Escherichia coli, and Shigella dysenteriae*) [17,24,25,47].

Anti-biofilm activity of *C. sativus* tepal and stigma extracts in biofilm producer staphylococcal reference strains has not been previously studied. The MICs of *C. sativus* stigma and tepal extracts were >512 µg GAE/mL against both *S. aureus ATCC* 43300 and *S. epidermidis ATCC* 35984. Our data demonstrated that *C. sativus* stigma and tepal extracts were able to interfere with biofilm formation by ATCC 43300 and by ATCC 35984. Interestingly, these inhibitions were observed for concentrations (64–128 µg GAE/mL) that were the lower MIC concentrations. This potential to inhibit biofilm formation without inhibiting bacterial growth is an interesting aspect as it may prevent the development of bacterial resistance while blocking bacterial adhesion, which is essential for surface colonization and infection.

ATCC 43300 and ATCC 35984 are two gram-positive pathogens that can cause serious human diseases. *S. aureus* can cause inflammation in the dermal mucous membranes and many other tissues and organs. *S. epidermidis* is involved in human skin disease and acne vulgaris. These bacterial pathogens form biofilms that are multicellular surface-attached communities of bacteria embedded in the extracellular matrix (ECM). Bacteria living in biofilms easily colonize the surfaces of certain medical devices. Biofilms allow embedded bacteria to resist antimicrobial therapy [27], such as reducing contact with antimicrobial compounds or reducing metabolic activity. The lower sensitivity to multiple antibiotics is an obstacle when treating biofilm-associated acute and chronic infections and caused a large number of problems in health care, food industry, and other fields [28]. The observed antibiofilm activity of *C. sativus* tepal and stigma extracts could be related to the bioactive molecules analyzed in this work, including phenolic compounds and terpenoids. This hypothesis is supported by previous studies which have demonstrated that biofilm formation is associated with oxidative stress [48]. In this regard, some phenolic terpenes (such as carvacrol, thymol, and eugenol) were found to present anti-biofilm potential against staphylococci [49] and were found in high abundance in both the analyzed extracts (Table S1).

Antimicrobial and anti-biofilm activities against *S. aureus, P. aeruginosa, E. coli, and S. epidermidis* of plant polyphenols, including flavonoids (mainly anthocyanins), have been previously observed and reviewed [18,21,50]. In this regard, polyphenols interact with both bacterial proteins, leading to their inactivation and loss of function, and with cell wall structures, inducing modification of physicochemical properties of membranes. It has also been reported that polyphenols can reduce the adhesion of bacteria to fibrinogen, which is the first step in the formation of *S. aureus* biofilms [51] and destroy the activity of enzymes involved in the formation of biofilms, such as sortase A, and thus, preventing the adhesion to surface proteins [51]. In addition, they are able to alter the synthesis of nucleic acid and the energy metabolism [21]. For example, flavonoids can inhibit the gene expression of adhesion-related surface proteins such as fibrinogen-binding proteins (ClfA and ClfB) and fibronectin-binding proteins (FnBPs) that are involved in the biofilm formation [51].

In conclusion, the formation of a biofilm is one of the mechanisms of antibiotic resistance in *S. aureus* and *S. epidermidis*. Due to their antioxidant properties, polyphenols can reduce oxidative stress-mediated virulence in pathogenic microorganisms by scavenging free radicals and/or other mechanisms. The antioxidant and anti-biofilm activities of extracts of *C. sativus* tepals and stigmas support further studies for valorizing high-quality by-products of saffron, which are usually discarded. *C. sativus* stigma and bio-residues such as tepals are antioxidant sources and good candidates as an effective agent in preventing biofilm formation that may have application in pharmaceutical industries and may be used for formulation of innovative functional foods.

Acknowledgments: The authors are grateful to local farm Stachys (Recanati, MC, Italy) and Azienda Lorenzini (Ancona, Italy) for providing the *C. sativus* flowers. The authors thank the "Romeo ed Enrica Invernizzi" foundation (Milan, Italy) for its kind support in providing the metabolomics facility at Università Cattolica del Sacro Cuore.

Funding information: This research received no external funding.

Author contributions: L.B., E.M., G.M., C.M.: methodology, formal analysis, investigation, and data validation; G.R.: methodology, formal analysis, and investigation; G.R. and E.M.: formal analysis, visualization, and writing – original draft; A.P.: review and editing; B.F. and L.L. supervision, writing – original draft, and review and editing; G.F. and T.B.: supervision, resources, writing – original draft, and review and editing.

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Ethical approval: The conducted research is not related to either human or animal use.

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