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RESEARCH ARTICLE

Antibiofilm, cellular antioxidant, anti-inflammatory, immunomodulatory, cytotoxic, and antimutagenic activities of soluble melanins from *Randia echinocarpa* fruit

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Abstract
Melanins are widely distributed biopolymers that exhibit important biological activities. However, fruit melanins have been scarcely studied. In this work, the antibiofilm, cellular antioxidant, anti-inflammatory, immunomodulatory, cytotoxic, and antimutagenic activities of soluble melanins (SMs) isolated from the *Randia echinocarpa* fruit (papache) were evaluated. The SMs inhibited biofilm formation in *Staphylococcus aureus* MDR and ATCC 43300 up to 60% at 1000 µg/mL; they presented a cellular antioxidant activity (60.02%) at 50 µg/mL, were immunomodulatory by increasing the peripheral blood mononuclear cells (PBMC) proliferation index (1.09 at 50 µg/mL), and inhibited HeLa cell proliferation by 77.39% (IC_{50} = 9.34 µg/mL). SMs were neither toxic nor mutagenic in the *Salmonella* Typhimurium YG1024 strain and inhibited the 1-nitropyrene mutagenicity by 30.2%. The biological activities of papache SMs support their potential to be used in nutraceutical and pharmaceutical formulations.

Keywords *Randia echinocarpa* · Antibiofilm activity · Cellular antioxidant activity · Immunomodulatory activity · Antitumor activity · Antimutagenic activity

Introduction

Randia echinocarpa Moc. and Sessé ex DC. (Rubiaceae) is native to Mexico, commonly known in the northwest State of Sinaloa as papache, used in Mexican traditional medicine to treat diverse diseases/symptoms (e.g., cancer, malaria, diabetes, peptic ulcers, and diseases of kidney, circulatory system, and lung), and several biological activities of the plant and its fruit have been described (e.g., antibacterial, nematicide, antioxidant, cicatrizing, antimutagenic, proliferative, diuretic, toxicity) (Ojeda-Ayala et al. 2022). The papache fruit presents a dark-flesh color due to melanins (Cuevas-Juárez et al. 2014; Montes-Avila et al. 2018).

Melanins are high molecular weight complex biomolecules formed by the oxidation and polymerization of phenolic or indolic molecules and found in various living organisms, including humans, animals, plants, and microorganisms. They are classified into animal eumelanin (black pigments) and pheomelanin (red and brown pigments), and plant allomelanin or phytomelanin (brown and black) (Glagoleva et al. 2020; El-Naggar and Saber 2022). Melanins have a wide range of biological activities, including photoprotective, antioxidant, free radical scavenging, anti-inflammatory, immunomodulatory, antimicrobial, and anticancer, and provide hepatic, gastrointestinal, and hypoglycemic benefits (El-Obeid et al. 2016). However, in comparison with those in animals and microorganisms, melanins in plants/fruits have been less studied (Hung et al. 2003; Huang et al. 2011; Cuevas-Juárez et al. 2014; Montes-Avila et al. 2018; Pío-León et al. 2018; Gil-Avilés et al. 2019; Al-Obeed et al. 2020; Alam et al. 2022).

Pure melanins are generally known for their insolubility. However, melanins in living organisms interact with other biomolecules, such as carbohydrates and proteins,

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increasing their solubility in polar solvents; such complexes are named soluble melanins (SMs) (Cuevas-Juárez et al. 2014). Papache fruit has SMs, and solubility is associated with the formation of conjugates of melanins with organic acids and carbohydrates (Montes-Avila et al. 2022). Moreover, the insoluble melanins of papache fruit present high antioxidant and immunomodulatory activities (Montes-Avila et al. 2018), whereas the SMs have high antioxidant and α-glucosidase inhibitory values (Cuevas-Juárez et al. 2014); besides, SMs are innocuous in Balb/C mice (Gil-Avilés et al. 2019). The aim of this study is to evaluate the antibiofilm, cellular antioxidant, anti-inflammatory, immunomodulatory, cellular cytotoxic, and antimutagenic activities of the soluble melanins of papache fruit. This characterization will contribute to establishing the potential of *R. echinocarpa* fruit as a source of soluble melanins to be used in nutraceutical and pharmaceutical formulations, which would help in the management and sustainable use of this floristic resource from Mexico.

Materials and Methods

Plant material

Ripe fruit of *Randia echinocarpa* Sessé et Mociño was collected from the municipalities of Culiacan, Badiraguato, and Salvador Alvarado, all in Sinaloa, Mexico. Dr. Rito Vega-Aviña, a professor at the Agronomy School of the Autonomous University of Sinaloa, identified the plant species. A voucher specimen deposited in the herbarium of the Agronomy School has the number 6307. The edible pulp of *R. echinocarpa* without woody peels and seeds was frozen and freeze-dried. The lyophilized sample was milled to obtain a powder that passed through a no. 40 sieve; then, it was stored at − 20 °C until use.

Extraction and purification of soluble melanins (SMs)

Soluble melanins were extracted as previously reported (Cuevas-Juárez et al. 2014), with minor modifications. Fruit powder (5 g) and boiling deionized water (100 mL) were mixed and stirred for 30 min. The suspension was centrifuged (20,000 × g/15 min/20–25 °C); the recovered supernatant was frozen at − 80 °C and freeze-dried. The residue named impure SMs (0.5 g) was dissolved in distilled water (10 mL) and dialyzed against 300 mL of deionized water using a 12-kDa cellulose membrane. The water was changed twice a day for 4 days. Dialyzed samples were recovered and freeze-dried to obtain the SMs.

Antibiofilm activity

Bacterial biofilm formation was assayed using the crystal violet method on 96-well flat-bottom polystyrene microplates. Human pathogenic bacteria classified as multidrug-resistant and sensitive with different biofilm formation capacities were used: two *Escherichia coli*, five *Staphylococcus aureus*, and one *Pseudomonas aeruginosa* strains; these bacteria were four clinical isolates and four reference ATCC strains (**Online Resource 1**).

Inhibition of biofilm formation

A fresh bacterial culture (TSA/37 ºC/18–20 h) was used to prepare a suspension in saline solution (0.85% w/v), adjusting its turbidity to the 0.5 McFarland standard (1×10^8 CFU/mL). The test inoculum (1×10^6 CFU/mL) was prepared in TSB medium + 1% glucose, and 100 µL were deposited in each well. SMs (100 µL) at different concentrations (1000, 500, 100, 50 µg/mL) were added before the inoculum. The microplates were incubated (37 °C/24 h), and planktonic bacteria were removed. Wells were washed thrice with PBS (250 µL, pH = 7.2) and dried in an inverted position. The adhered bacteria were fixed with methanol (150 µL, 15 min), stained with 1% crystal violet (150 µL, 15 min), the dye solution was removed, and the dye in excess was eliminated with three washes of sterile distilled water (250 µL). Once the microplate was dry, the dye adhered to the formed biofilm was eluted with 95% ethanol (150 µL), and the optical density (OD) at 540 nm was determined (Dolatabadi et al. 2018). The following controls were employed: growth, inoculum and solvent; blank, medium with extract; and sterility, medium with solvent. Each evaluation was performed in quadruplicate in three independent tests.

Biofilm eradication

Bacterial biofilm was formed (37 °C/24 h) as previously described. Then, non-adherent bacteria were removed by washing with PBS (750 µL), 200 µL of SMs at different concentrations (2000, 1000, 200, 100 µg/mL) were added to the formed biofilm, and it was incubated again (37 °C/24 h). Subsequently, washing (PBS), fixation (methanol), crystal violet staining, biofilm elution (95% ethanol), and determination of optical density (OD) at 540 nm (Dolatabadi et al. 2018) were carried out. The following controls were employed: growth, inoculum

with solvent; blank, medium with extract; and sterility, medium with solvent. Three independent experiments were performed in quadruplicate (Mohammadi et al. 2019).

Biofilm metabolic activity

The metabolic activity of the biofilms formed in the absence and presence of SMs was determined using the XTT reduction assay. Biofilms were formed as described above and washed with PBS (150 µL) to remove non-adherent bacteria after and before SMs addition (2000–50 µg/mL) for biofilm inhibition and biofilm eradication assays, respectively. Once dry, 100 µL of PBS and 20 µL of the XTT-PMS 1% solution (1 mg/mL) were added to each well of the plate. The plates were incubated in the dark (37 °C/3 h), and the absorbance of the reaction mixture was measured at 450 nm. The employed controls were the following: growth, inoculum with solvent; blank, medium with extract; and sterility, medium with solvent. Each assay was performed in duplicate in two independent assays (Costa et al. 2017).

Light microscopy of biofilm

The effect of SMs on inhibiting and eradicating bacterial biofilms was observed under an optical microscope. The test inoculum was added to 24-well polystyrene plates containing 400 µL TSB medium + 1% glucose and sterile round glass coverslips (12 mm) in the absence and presence of SM at different concentrations (2000–50 µg/ mL). The plates were incubated (37 ºC/24 h), and the biofilms adhered to the coverslips were washed and stained as previously described. Biofilms were visualized at 40X (Ali et al. 2016; Kannappan et al. 2017; Qais et al. 2019).

Cellular antioxidant activity in red blood cells (CAA-RBC)

The antioxidant activity of SM was evaluated according to the methodology reported by Blasa et al. (2011) with some modifications. A blood sample (3 mL) from apparently healthy volunteer donors was centrifuged (10 min/2500 rpm). The red blood cells (RBC) were recovered, washed, and resuspended with PBS (pH 7.4, 4 °C, 1:20 v/v). The RBC suspension was incubated (37 °C/200 rpm/ 30 min) with 250 µL DCFH-DA (140 µM in PBS) and 250 µL SMs (50 µg/mL) or quercetin standard (10 µM); the mixture was centrifuged (2500 rpm/10 min), and the pellet was washed and diluted (1:10 v/v) with PBS (pH 7.4, 4 °C). Subsequently, 200 µL of the cell suspension and 50 µL of AAPH (500 µM) were mixed, and fluorescence (485 nm ex and 538 nm em) was measured every 2 min for 10 h. Two assays were performed in triplicate, including a control (no samples) and a blank (no samples or AAPH). The antioxidant capacity (CAOx) of the samples was calculated with the formula: CAOx = 100- (∫SA/∫CA) × 100, where ∫SA is the integration of the area under the curve of the sample and ∫CA is the integration of the area under the curve of the control (Blasa et al. 2011). The apparently healthy volunteer donors gave informed consent before being included in the study.

In vitro anti-inflammatory activity

The human red blood cell (HRBC) membrane stabilization assay was used (Yesmin et al. 2020). Anti-inflammatory activity was determined by measuring the ability of SMs (400 µg/mL) to prevent hemolysis induced by heat or by a hypotonic medium. Diclofenac sodium (25–1500 µg/mL) was used as a positive control. The percentage of inhibition of hemolysis was calculated according to the equation: % inhibition of hemolysis $= 100 \times \left([1] - \left[\frac{OD2-OD1}{OD3-OD1}\right]\right)$. where: OD1 = Optical density of the unheated/isotonic sample, OD2 = Optical density of the heated/hypotonic sample, and OD3 = Optical density of the heated/hypotonic control. Samples were tested at concentrations that did not cause hemolysis. Two independent experiments were performed in triplicate.

In vitro immunomodulatory activity

The model of peripheral blood mononuclear cells (PBMC) was used. PBMC were isolated by density gradient from human blood samples of apparently healthy volunteers according to Goyal et al. (2019) and Jenny et al. (2011) with slight modifications. The recovered cells were suspended in 1 mL of RPMI-1640 medium supplemented with 10% (v/v) of FBS and 0.5% (v/v) of antibiotic–antimycotic. PBMC at a density of 2×10^5 cells/well were treated with SMs (50 and 100 µg/mL), negative control (DMSO at 10% v/v), or positive control (10 µg/mL, phytohemagglutinin PHA or lipopolysaccharide LPS). The mixtures were incubated in a humidified atmosphere (37 °C/5% CO_2/24 h), centrifuged (1500 rpm/5 min), and the supernatant was removed. Cell proliferation was measured by the MTT method, reading the absorbance at 540 nm. Cell growth was reported as the proliferation index according to the formula: Proliferation index = (A/B). Where: A and B are the absorbance values for the treated and untreated cells, respectively. Two independent experiments were performed in triplicate.

Cellular cytotoxic activity

HeLa cervical cancer cells were used following the methodology reported by Malich et al. (1997) with some

modifications. Cells were grown in DMEM medium until 90–95% confluency. Cell viability was determined using trypan blue dye (4% w/v). In a 96-well flat-bottom microplate, HeLa cells (12×10^3 cells/well) were incubated (37 °C/5% CO_2/24 h) in DMEM medium supplemented with FBS (10% v/v). Subsequently, SMs were added at different concentrations (1–500 µg/mL), and the controls were Paclitaxel (positive) and DMSO 10% v/v (negative). The microplate was incubated (37 °C/5% CO_2/72 h), and 100 µL of MTS (5 mg/mL in DMEM) were added to the monolayer cells. The microplate was incubated again (40–60 min), and the absorbance was measured at 492 nm. The results were expressed as a mortality percentage and mean lethal concentration (LC_{50}). Two independent experiments were performed in triplicate.

Antimutagenic activity

The microsuspension assay of the Ames test was used (Kado et al. 1983). *Salmonella enterica* serovar Typhimurium YG1024 was the tester strain, and 1-NP was the mutagen. Toxicity and mutagenicity were evaluated for SMs. Tester strain YG1024 was grown overnight (37 °C) in Oxoid Nutrient Broth No. 2 to approximately $1–2 \times 10^9$ cells/mL and harvested by centrifugation (3000 × g/4 °C/10 min). Bacteria cells (1×10^{10} cells/mL) were resuspended in ice-cold PBS (0.15 M, pH 7.4) and ingredients were added in the following order to 10 × 100 mm sterile glass culture tubes kept on ice: 0.1 mL cocktail, 0.1 mL of bacteria (1×10^{10} cells/mL PBS), 0.01 mL 1-NP (20 ng/tube) or 0.005 mL of the SM (50 µg/tube), and 0.005 mL 1-NP (20 ng/tube). The mixture was incubated (37 °C/90 min) in the dark with vigorous shaking. The tubes were placed in an ice bath, removed one at a time, and 2 mL of molten top agar containing 90 nmol histidine/biotin was added. The combined solutions were vortex mixed and poured onto medium minimal Vogel-Boner plates. Plates were incubated (37 °C/48 h) in the dark, and the colonies were counted. Samples were tested in triplicate for two independent experiments. Strain markers and bacterial survival were routinely monitored for each experiment. The mutagen and all samples were dissolved in DMSO. Samples were sterilized by filtration (0.22 µm). Mutagenic index (MI) was calculated as the ratio of induced revertants by the evaluated substance to spontaneous revertants. The SMs were considered mutagenic if MI ≥ 2 and cytotoxic if MI ≤ 0.6 (Maron et al. 1983; Fernandes et al. 2003; Moreira-Rosa et al. 2006). The antimutagenic activity was calculated as the inhibition percentage of mutagenic activity, % inhibition = (1 − A/B) × 100. Where: A = number of revertants per plate in the presence of SMs and mutagenic agent, and B = number of revertants per plate in the presence of the mutagenic agent.

Statistical analysis

All data were expressed as the means ± SD. They were analyzed by one- or two-way ANOVA, and significant differences ($P \leq 0.05$) between the means were established by the Fisher or Bonferroni posttest. Also, a Probit analysis was performed. The statistical programs STATGRAPHICS plus version 5.1 (Statistical Graphics Corporation™, USA), GraphPad Prism version 8.0.1 (GraphPad Software, USA), and Excel (Microsoft Corporation, USA) were used.

Results and discussion

Antibiofilm activity

The SMs of papache fruit inhibited biofilm formation in *S. aureus* ATCC 43300, 3R, 4R, and 5R strains by more than 50% and up to 60% at a concentration of 1000 µg/mL ($P < 0.05$). At lower concentrations of SMs (100, 50 µg/mL), an opposite effect was observed, with an increase in biofilm formation of up to 3 times in the clinical isolates (3R, 4R, and 5R) and 1.6 times in the reference strains (ATCC 43300 and 25,923) (Fig. 1). The antibiofilm activity of melanins, mainly of bacterial origin, has been previously reported. Melanin pigments (100 µg/mL) from actinobacteria *Nocardiopsis* inhibited up to 65.9% of the formation of *Staphylococcus* sp. biofilm (Kamarudheen et al. 2019). Melanins (100 µg/mL) from *Vibrio alginolyticus*, *Pseudomonas stutzeri*, and *Providencia rettgeri* reduced 37%-89.08% the formation of biofilms from foodborne

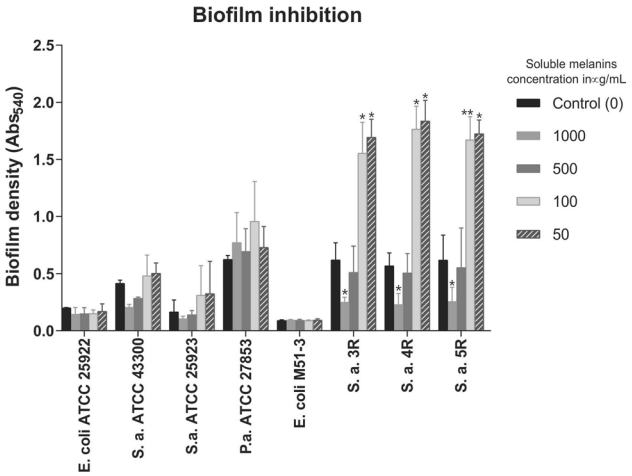

Fig. 1 Optical density (OD) values in the evaluation of the inhibition of bacterial biofilm by papache soluble melanin (50–1000 µg/mL). The mean ± SD of three independent experiments with four replicates is plotted. * $P < 0.05$ versus the control group, employing the Bonferroni Test. (S.a. = *Staphylococcus aureus*; P.a. = *Pseudomonas aeruginosa*)

strong biofilm-producing bacteria (*Bacillus altitudinis*, *Pseudomonas aeruginosa*, *Staphylococcus warneri*, and *Bacillus* sp.) (Kurian et al. 2015; Laxmi et al. 2016; Kurian et al. 2018). On the other hand, nanomelanins (30 µg/mL) of *Pseudomonas* sp. inhibited 71% of the formation of the *S. aureus* biofilm (Kiran et al. 2017). Regarding melanins of fungal origin, it was reported that *Auricularia auricula* melanin (80 µg/mL) inhibited biofilm formation of *E. coli* K-12, *P. aeruginosa* PAO1, and *P. fluorescens* up to 71.3, 61.7, and 63.2%, respectively, in a concentration-dependent manner (Bin et al. 2012). In the case of soluble melanins, Wang et al. (2019) reported that melanin pigments (512 µg/mL) from *Streptomyces* sp. showed antibiofilm activity against *S. aureus* and *P. aeruginosa* with inhibition percentages of up to 67.5% and 74.6%, respectively, generally resulting in lower inhibition percentages than insoluble melanins (79.2%-71.7%). Previous studies with soluble melanins from sources different from fruits showed inhibition rates of bacterial biofilm formation similar to those of papache SMs (Fig. 1). However, their concentrations were 10 times lower than those used in the present study. It is worth mentioning that most of the antibiofilm activity studies have used melanins from bacteria or fungi, some of them edible. Meanwhile, SMs isolated from the *R. echinocarpa* fruit were evaluated in the present work.

Regarding the *E. coli* strain ATCC 25922 (weak biofilm former), there was a non-significant inhibition in the biofilm formation capacity at all SMs concentrations compared to the control. The clinical isolate *E. coli* M51-3 (non-biofilm-forming) was not affected by the SMs. In the case of *P. aeruginosa* ATCC 27853 (strong biofilm former), there was a non-significant increase in generalized biofilm formation compared to the control at all SMs concentrations evaluated (Fig. 1). These results in Gram-negative bacteria agree with those reported for melanin pigments (100 µg/mL) from actinobacteria *Nocardiopsis*, where no effect was observed on *P. aeruginosa* (Kamarudheen et al. 2019). However, the results contrast with those reported from bacteria (*Vibrio alginolyticus*, *Pseudomonas stutzeri*, *Providencia rettgeri*, and *Streptomyces* sp.) and melanin of the fungus *Auricularia auricula* with inhibition percentages of 56% to 79% (Bin et al. 2012; Kurian et al. 2015; Laxmi et al. 2016; Kurian et al. 2018; Wang et al. 2019). Also, they contrast with the in silico study of Venkatesh et al. (2018), where human oral mucosa containing melanin presented antibiofilm properties by acting as an antagonist of quorum sensing in *P. aeruginosa* containing protein LasA. Previously, SMs of papache did not inhibit the growth of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 up to 5000 µg/mL (Montes-Avila et al. 2022); therefore, the evaluations performed in the present study were at sub-inhibitory concentrations (< 2000 µg/mL). It has been reported that the formation of biofilm is induced by exposing bacteria to sub-inhibitory concentrations of various antimicrobials, which is related to changes in their genotype and phenotype caused by the stress, as well as the release of the contents of the dead sensitive microbial cells that which can act as a substrate for biofilm formation (Kaplan 2011; Bernardi et al. 2021). This induction of biofilm formation is similar to that observed using lower concentrations (100 and 50 µg/mL) of papache SMs.

The SMs of papache do not affect the bacterial viability in the biofilm, as demonstrated in the case of *E. coli* and *P. aeruginosa*, where no significant effect on biofilm inhibition was observed, evidenced by the crystal violet staining method (**Online Resource 2**). However, the microscope visualization of the formed biofilms allowed us to appreciate the effect of the papache SMs in inhibiting the biofilm and bacterial viability. An increase in biofilm formation was observed in the *S. aureus* strains at low concentrations of SMs and an inhibition effect at higher concentrations, clearly present in the ATCC 43300, 3R, 4R, and 5R strains that share the characteristic of being MRSA compared to the non-MRSA strain ATCC 25923 (Fig. 2). This behavior has been reported when comparing resistant and sensitive strains or clinical isolates and reference strains (Piechota et al. 2018; Mahmoudi et al. 2019; Hosseini et al. 2020; Leshem et al. 2022), indicating that the ability to form a biofilm is a resistance mechanism of the strains to survive in the presence of some antimicrobial since the structure and characteristics of the biofilm formed provides protection: e.g., extracellular polymeric substances (EPS) reduce the penetration of antimicrobials, the bacterial growth rate is reduced, presence of persistent cells, activation of genes related to biofilm protection (Singh et al. 2021). In the case of *E. coli* strains, no effect on M51-3 and a slight decrease in ATCC 25922 biofilm viability was observed, which corresponded with the biofilm inhibition results. Regarding *P. aeruginosa*, non-significant increases in biofilm formation were observed compared to the control at all concentrations tested (Fig. 2, **Online Resource 3**).

In brief, no biofilm eradication effect was observed for any SMs concentrations evaluated. In contrast, SMs promoted biofilm formation by the strains *S. aureus* and *P. aeruginosa*, resulting in more biofilm at higher concentrations (2000, 1000 µg/mL). In the case of *E. coli*, there are no relevant changes in eradication (Fig. 3), corroborated by the metabolic activity evaluation (**Online Resource 4**) and the light microscope images (Fig. 4, **Online Resource 5**).

Cellular antioxidant activity

The SMs showed a cellular antioxidant activity (60.02%) similar to that of the quercetin control (62.86%); however, the concentration of quercetin evaluated was 16.6 times lower than SMs (Table 1). Previous studies have shown

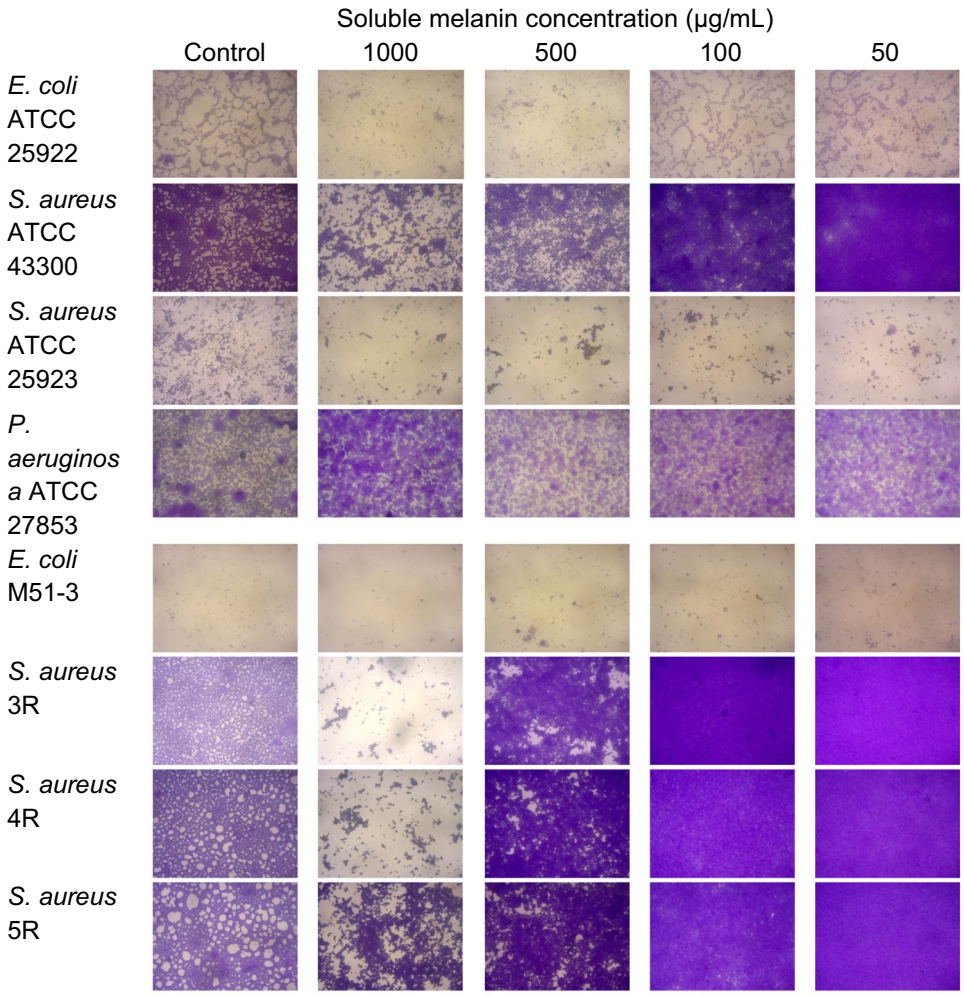

Fig. 2 Optical microscopy (40X) of the effect of papache soluble melanins in the inhibition of bacterial biofilm formation (Carl Zeiss microscope Primostar model)

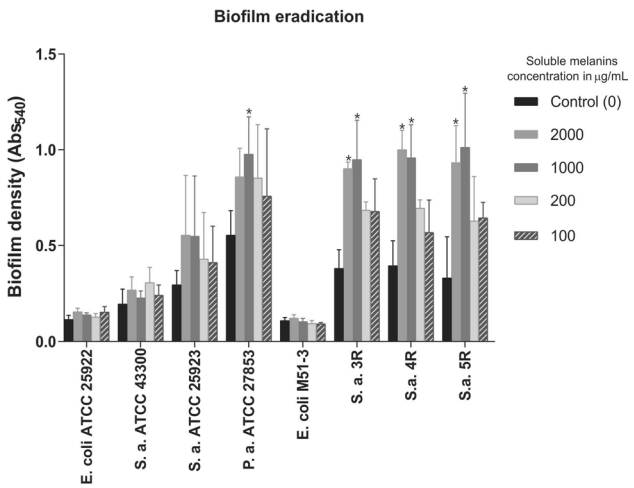

Fig. 3 Optical density (OD) values in the evaluation of the eradication of bacterial biofilm by papache soluble melanin (100–2000 µg/mL). The mean ± SD of three independent experiments with four replicates is plotted. $*P < 0.05$ versus the control group, employing the Bonferroni Test. (S.a. = *Staphylococcus aureus*; P.a. = *Pseudomonas aeruginosa*)

the good antioxidant activity in vitro of *R. echinocarpa* melanins (µmol TE/g): the insoluble melanins have higher values (ABTS = 835.7; FRAP = 709.64) than those of the soluble melanins (ABTS = 719.5; FRAP = 351.77). Besides, low concentrations of insoluble melanins (10 and 100 µg/mL) protect *Saccharomyces cerevisiae* from H_2O_2 damage (Cuevas-Juárez et al. 2014; Montes-Avila et al. 2018). Similarly, melanins of fungi (Arun et al. 2015; Barretto et al. 2020; Ben Tahar et al. 2020; Wold et al. 2020; Xu et al. 2020; Oh et al. 2021; Li et al. 2022; Surendirakumar et al. 2022), bacteria (Kurian et al. 2015; Wang et al. 2019; Bayram et al. 2020; Ferraz et al. 2021) and fruits (Cuevas-Juárez et al. 2014; Montes-Avila et al. 2018; Pío-León et al. 2018; Alam et al. 2022) show very good antioxidant activity in vitro. However, the evidence in cellular models is scarce; melanins isolated from *Sporisorium reilianum* (200–400 µg/mL) protected HepG2 cells from H_2O_2-induced oxidative damage by reducing ROS, MDA, and LDH levels (Fu et al. 2022); *Auricularia auricula* melanins (1.2 mg/mL) protected L02 cells by inhibiting the increase in

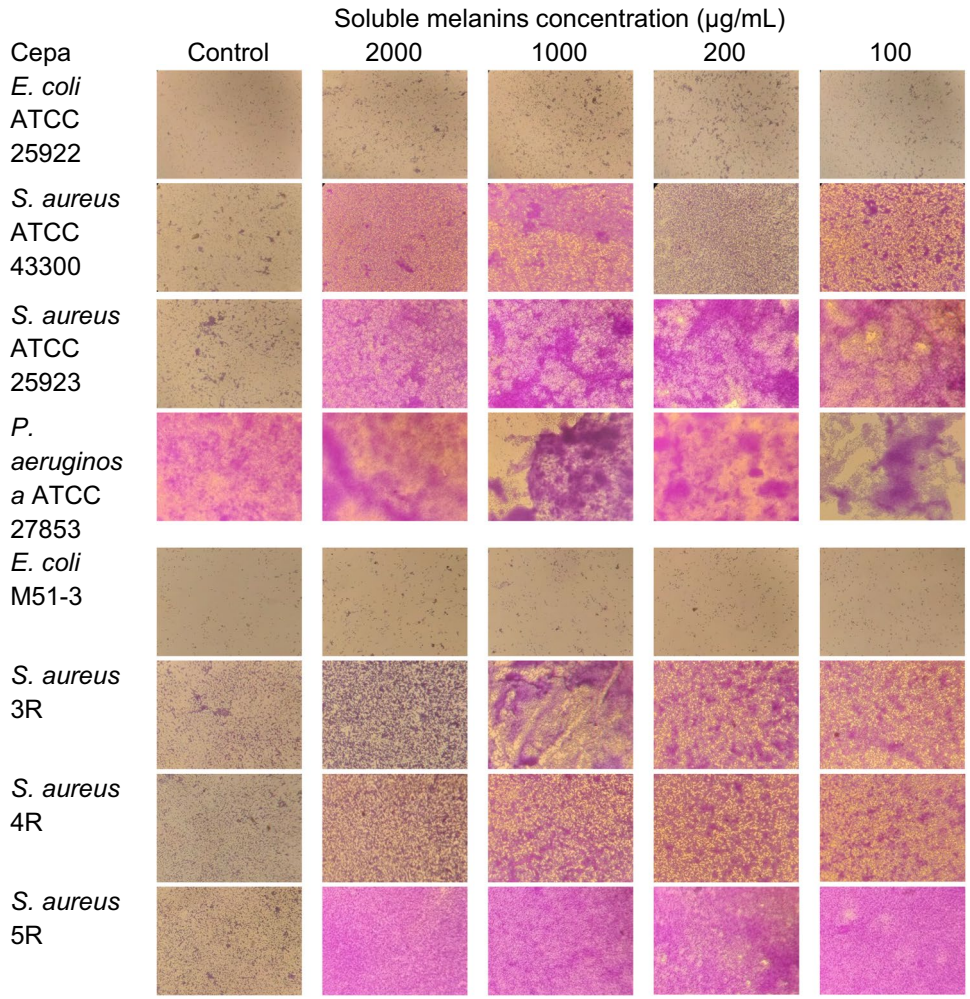

Fig. 4 Optical microscopy (40X) of the effect of papache soluble melanins in the eradication of bacterial biofilm (NIKON Eclipse model E200 microscope)

Table 1 Biological activities of soluble melanins obtained from papache fruit

Biological Activity	Soluble Melanins	Control
Cellular antioxidant (%) a	60.02 ± 0.75	62.86 ± 0.35
Anti-inflammatory (IC50, µg/mL) b	NA	118.5 ± 4.31 (Heat)/ 147.6 ± 5.66 (Hypotonic)
Immunomodulatory (proliferation index) c	1.09 ± 0.12 / 1.10 ± 0.18	1.74 (PHA), 0.67 (LPS)
Cellular cytotoxic (IC50, µg/mL) d	9.34	2.56f
Antimutagenic (inhibition %) e	30.2 ± 7.09	-

aSM evaluated at 50 µg/mL and quercetin control at 3.01 µg/mL (10 µM)

bNA: no activity; SM evaluated at 400 µg/mL and diclofenac sodium control at 25–1500 µg/mL

cSM evaluated at 50 and 100 µg/mL and PHA and LPS controls at 10 µg/mL

dSM evaluated from 1–500 µg/mL. f Paclitaxel IC50 = 8 nM, 2.56 ng/mL

eSpontaneous revertants: 19.7 ± 1.53 revertants/plate

ROS induced with alcohol (Hou et al. 2021) and from oxidative injury induced by H_2O_2 (1.6 mg/mL) (Liu et al. 2019). Although the cellular models used are different, it is evident that melanins have good cellular antioxidant activity, highlighting the papache SMs that showed a good percentage of antioxidant capacity at concentrations considerably lower than those reported by other authors. In the evaluation carried out, SMs penetrate the erythrocyte and inactivate peroxyl radicals, an effect related to the characteristic of scavenging and quenching

of superoxide anions and singlet oxygen species, as well as to the reducing capacity of the phenol-quinone structure of melanins (Cuevas-Juárez et al. 2014; Montes-Avila et al. 2018; Hou et al. 2021; Alam et al. 2022; Fu et al. 2022).

In vitro anti-inflammatory activity

The SMs of papache were inactive in stabilizing the red blood cell membrane against heat-induced stress or hypotonicity up to the highest concentration tested (400 µg/mL). However, the anti-inflammatory activity of melanins evaluated has been demonstrated using other methods. Bacterial melanins (100 µg/mL) from *Vibrio alginolyticus* BTKKS3, *Pseudomonas stutzeri* BTCZ10, *Providencia rettgeri* BTKKS1, and *Bacillus* spp. BTCZ31 inhibited in a dose-dependent manner the expression of inflammatory enzymes (*i.e.*, COX, LOX, MPO, and NO synthase) in RAW 264.7 cells (Kurian et al. 2015; Kurian et al. 2015; Kurian et al. 2017; Kurian et al. 2018). Similarly, the soluble melanins of *Bacillus thuringiensis* subsp. galleries K1 decrease in RAW 264.7 cell line the levels of nitric oxide, prostaglandin E2, and proinflammatory cytokines, including interleukin IL-6 and IL-1b (Petrosyan et al. 2019). On the other hand, melanins produced by the yeast *Nadsoniella nigra* reduced the content of IL-1b in rat serum and restored the level of anti-inflammatory cytokines (IL-10, TGF-b) to the control values (Belemets et al. 2017). Moreover, melanins isolated from *Inonotus obliquus* reduced nitric oxide production in primary murine macrophages (Wold et al. 2020). Similarly, the melanins extracted from *Cryptococcus rajasthanensis* have anti-inflammatory activity in the inhibition assay of albumin protein denaturation (IC_{50} = 40.50 µg/mL) (Barretto et al. 2020). Besides, the topical treatment with a 2.5% solution of the herbal *Nigella sativa* melanin inhibits the swelling induced by formalin in the rat-paw edema (El-Obeid et al. 2016). Thus, there is good evidence of the anti-inflammatory activity of soluble and insoluble melanins from different origins, which is attributed to the presence of phenolic groups and their antioxidant and free radicals scavenging actions that can help in reducing the inflammatory response (El-Obeid et al. 2016; Belemets et al. 2017; Barretto et al. 2020). Despite the good antioxidant activity of papache SMs, they did not show anti-inflammatory activity in the assay tested, and it is necessary to evaluate this activity using other methods.

In vitro immunomodulatory activity

The PBMC proliferation indices of the SMs (1.09 at 50 µg/mL and 1.10 at 100 µg/mL) were lower than that of the PHA control (1.74) but higher than that of LPS (0.67) (Table 1). The immunomodulatory activity of insoluble melanins (50 µg/mL) from *R. echinocarpa* was previously reported in mouse splenocytes with a proliferation index of 1.37 (Montes-Avila et al. 2018), a value higher than that reported for SM of the same fruit in the present work. The enhancement and modulation of the immune system by natural melanins have been reviewed (ElObeid et al. 2017). Likewise, it was reported that *Lachnum singerianum* melanins, modified with histidine to increase their solubility, repressed tumor growth through activation of the immune response, improved the levels of SOD, CAT, IL-2, and TNF-α, and reduced the level of MDA (Ye et al. 2019). On the other hand, solubilized synthetic melanins suppressed the cytokine and reactive oxygen species production in macrophages stimulated by fungal components (Tajima et al. 2019). Moreover, *Nigella sativa* melanins stimulate IL-1β production in monocytes and THP-1 cells (El-Obeid et al. 2021). Immunomodulation is a broad term that refers to any changes in the immune response. It may involve induction, expression, amplification, or inhibition of any part or phase in the immune response (Venkatalakshmi et al. 2016). Melanins have both an immunostimulatory and an immunosuppressive effect, which has allowed them to be considered a promising tool in cancer therapy and may be adjuvants in treating other chronic diseases (Cuzzubbo et al. 2021; Marcovici et al. 2022). According to Liu et al. (2021), melanin interacts with the immune system through diverse pathways, reducing the effectiveness of phagocytic cells, binding effector molecules and antifungals, and modifying complement and antibody responses. Some plant phytochemicals, including some from fruits, have shown potential immunostimulant activity (Venkatalakshmi et al. 2016; Maheshwari et al. 2022). According to the results of the present study, the SMs could be considered immunomodulatory agents, specifically immunostimulatory; an activity associated with the phenolic groups in the melanin structure (Gil-Avilés et al. 2019).

Cellular cytotoxic activity (antitumor activity on human cancer cell lines)

The SMs inhibited HeLa cell proliferation by 77.39% at 100 µg/mL (IC_{50} = 9.34 µg/mL) (Table 1). Similar results have been reported for insoluble melanins from *Streptomyces parvus* (20–5 µM), reaching values in the range of 14.28%-97.14% (IC_{50} = 10 µM) (Bayram et al. 2020). On the other hand, the results of SMs of papache contrast with those reported for insoluble melanins of *Pseudomonas putida* and *Sepia officinalis* that did not show a cytotoxic effect against HeLa-Kyoto cells (IC_{50} = 2.5 mg/mL), considering 0.1 mg/mL the established limit of toxicity for human cell lines for a mixture of natural compounds (Ferraz et al. 2021). Several studies of fungal and bacterial melanins have shown their cytotoxic effect on other carcinoma cell lines

(e.g., A549, SK-MEL-28, DLA, EAC, HFB4, Hep2 cells) (Arun et al. 2015; El-Naggar et al. 2017; Barretto et al. 2020; Surendirakumar et al. 2022). Moreover, other reports have demonstrated that melanins are non-toxic substances against various normal non-cancerous cell lines (e.g., NIH3T3, HaCaT, WI-38, WISH, CRL-1696b, L929, HUVEC) (Kurian et al. 2015; de Cassia Ribeiro Goncalves et al. 2016; El-Naggar et al. 2017; Kurian et al. 2017; Kurian et al. 2018; Ben Tahar et al. 2020; Liu et al. 2020). Regarding the cytotoxicity of soluble melanins, Hou et al. (2021) reported that *Auricularia auricula* melanins (1.6 mg/mL) are non-cytotoxic in normal human liver cells (L02). Besides, Wold et al. (2020) show that melanins of *Inonotus obliquus* are innocuous ($IC_{50} > 50$ µg/mL) in the human cancer cell lines NCI-H460 (lung carcinoma) and HT29-MTX (methotrexate-resistant colon adenocarcinoma). In the same context, soluble melanin produced by *Streptomyces glaucescens* showed potent cytotoxic activity against the HFB4 skin cancer cell line (81.3% at 100 µg/mL; $IC_{50} = 16.34$ µg/mL) and less cytotoxicity against normal non-cancerous-cells (human lung fibroblast WI-38, $IC_{50} = 37.05$ µg/mL; human amnion WISH, $IC_{50} = 48.07$ µg/mL) (El-Naggar et al. 2017). In the case of melanins obtained from plants or fruits, herbal melanins isolated from seed coats of *Nigella sativa* inhibited the proliferation of the colorectal adenocarcinoma HT29 and mCRC SW620 cell lines suggesting herbal melanins as a promising natural-based drug for the treatment of colorectal cancer (Al-Obeed et al. 2020).

According to El-Abd et al. (2022), compounds with IC_{50} values (µg/mL) in the ranges of 1–10, 11–20, 21–50, and 51–100 have very strong, strong, moderate, and weak cytotoxicity activity, respectively. Therefore, SM were strong cytotoxic agents ($IC_{50} = 9.34$ µg/mL) and can be considered possible natural agents for treating cancer as antitumor agent and used as a delivery nanoplatform (Cuzzubbo et al. 2021; Marcovici et al. 2022). In addition, papache SMs are harmless in acute and subacute toxicity tests in Balb/C mice (Gil-Avilés et al. 2019) and against *Artemia salina* (Montes-Avila et al. 2022). However, Andoh et al. (2022) recently mentioned that the safety of melanin as a food additive should be re-evaluated because it inhibits the growth of silkworm larvae.

Antimutagenic activity

The antimutagenic properties of medicinal plants are important for discovering new and effective preventive agents for mutations-related diseases (Makhafola et al. 2016). The SMs (50 µg/tube) showed a mutagenicity index (MI) of 0.78 ± 0.089 (spontaneous revertants/plate $= 18.3 \pm 0.58$) in the *S.* Typhimurium YG1024 strain. Thus, SMs were neither toxic ($MI \leq 0.6$) nor mutagenic ($MI \geq 2.0$) and presented a 30.2% inhibition of the 1-NP mutagenicity. There are few reports about the mutagenic/antimutagenic activity of melanins. de Cassia Ribeiro Goncalves et al. (2016) reported that melanins obtained from *Aspergillus nidulans* (1 g/plate) are not mutagenic in *S.* Typhimurium strains (TA97a, TA98, TA100, and TA102), which agrees with our results. On the other hand, Jong-Kyu et al. (1995) reported that *Bacillus licheniformis* SSA3 synthesizes a water-soluble dark brown melanin-type pigment, which inhibits up to 41% of the mutagenicity (10 µg/plate) induced by aflatoxin B1 in *S.* Typhimurium TA100 strain. Likewise, tea melanins have been reported to protect DNA from hydrazine-induced damage (Hung et al. 2003). Regarding the *Randia echinocarpa* fruit, it was previously shown that an aqueous extract inhibits the mutagenicity of 1-NP on *S.* Typhimurium YG1024 strain, acting by desmutagenic (damage prevention) and bioantimutagenic (damage repair) mechanisms (Santos-Cervantes et al. 2007); later, it was demonstrated that a methanol extract was an antimutagen in the same model, demonstrating that β-sitosterol and the acids linoleic and palmitic are the responsible compounds (Cano-Campos et al. 2011). In this regard, antioxidants can remove ROS before these react with DNA and result in a mutation (Sloczynska et al. 2014). This phenomenon corresponds with the correlation reported between antioxidant activity and the phenolic compounds content with the antimutagenicity of plant extracts (Makhafola et al. 2016). Papache SMs showed good antioxidant activity associated with their phenol-quinone structure, which could also be related to antimutagenic activity.

Conclusion

This study evaluated the antibiofilm, cellular antioxidant, anti-inflammatory, immunomodulatory, cellular cytotoxic, and antimutagenic activities of the soluble melanins of papache fruit. The obtained results demonstrate that the soluble melanins of papache fruit present biological activities relevant to human health: inhibits biofilm formation in multidrug-resistant bacteria; as an antioxidant in red blood cells, penetrate the erythrocyte and inactivate peroxyl radicals; increases the peripheral blood mononuclear cells proliferation index being immunomodulator; inhibits HeLa cell proliferation; and are not either toxic or mutagenic in the Ames assay. These characteristics make the soluble melanins of papache fruit a potential ingredient for nutraceutical and pharmaceutical formulations to prevent or treat chronic degenerative diseases. These findings should support the sustainable management and utilization of *R. echinocarpa* fruit. However, future studies should further

investigate the biological activities of soluble melanins of papache in vivo.

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Author contribution YPAS, GLA, RMPG, and FDV contributed to the study conception and design, set up the experiments, and analyzed the data; GLA and FDV also supervised the work. AFCS and JALV assisted in setting up experiments and writing the manuscript. YPAS and FDV wrote the first draft of the manuscript. All authors read and commented on the different manuscript versions and approved the final one.

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Data availability statement All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Ethical statement The human blood was collected from healthy volunteers following the Guidelines of venous blood specimen collection of the Clinical Laboratory Analysis of the Autonomous University of Sinaloa under official regulations governing the Health System in Mexico and with the approval of the local Ethics Committee of the Autonomous University of Sinaloa (Approval Number 22/0421).

Conflict of interest Yesmi P. Ahumada-Santos has no conflict of interest. Gabriela López-Angulo has no conflict of interest. Rebeca M. Pinto-González has no conflict of interest. Aldo F. Clemente-Soto has no conflict of interest. José A. López-Valenzuela has no conflict of interest. Francisco Delgado-Vargas has no conflict of interest.

References

Alam MZ, Ramachandran T, Antony A et al (2022) Melanin is a plenteous bioactive phenolic compound in date fruits (*Phoenix dactylifera* L.). Sci Rep 12(1):6614. https://doi.org/10.1038/s41598-022-10546-9

Ali K, Dwivedi S, Azam A et al (2016) *Aloe vera* extract functionalized zinc oxide nanoparticles as nanoantibiotics against multi-drug resistant clinical bacterial isolates. J Colloid Interface Sci 472:145–156. https://doi.org/10.1016/j.jcis.2016.03.021

Al-Obeed O, El-Obeid AS, Matou-Nasri S et al (2020) Herbal melanin inhibits colorectal cancer cell proliferation by altering redox balance, inducing apoptosis, and modulating MAPK signaling. Cancer Cell Int 20:126. https://doi.org/10.1186/s12935-020-01206-x

Andoh V, Chen L, Zhu F et al (2022) The evaluation of the biological effects of melanin by using silkworm as a model animal. Toxins (basel) 14(7):421. https://doi.org/10.3390/toxins14070421

Arun G, Eyini M, Gunasekaran P (2015) Characterization and biological activities of extracellular melanin produced by *Schizophyllum commune* (Fries). Indian J Exp Biol 53(6):380–387

Barretto DA, Vootla SK (2020) Biological activities of melanin pigment extracted from *Bombyx mori* gut-associated yeast *Cryptococcus rajasthanensis* KY627764. World J Microbiol Biotechnol 36(10):159. https://doi.org/10.1007/s11274-020-02924-0

Bayram S, Dengiz C, Gerçek YC et al (2020) Bioproduction, structure elucidation and in vitro antiproliferative effect of eumelanin pigment from *Streptomyces parvus* BSB49. Arch Microbiol 202(9):2401–2409. https://doi.org/10.1007/s00203-020-01956-2

Belemets N, Kobyliak N, Virchenko O et al (2017) Effects of polyphenol compounds melanin on NAFLD/NASH prevention. Biomed Pharmacother 88:267–276. https://doi.org/10.1016/j.biopha.2017.01.028

Ben Tahar I, Kus-Liśkiewicz M, Lara Y et al (2020) Characterization of a nontoxic pyomelanin pigment produced by the yeast *Yarrowia lipolytica*. Biotechnol Prog 36(2):e2912. https://doi.org/10.1002/btpr.2912

Bernardi S, Anderson A, Macchiarelli G et al (2021) Subinhibitory antibiotic concentrations enhance biofilm formation of clinical *Enterococcus faecalis* Isolates. Antibiot. https://doi.org/10.3390/antibiotics10070874

Bin L, Wei L, Xiaohong C et al (2012) *In vitro* antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom. Ann Microbiol 62(4):1523–1530. https://doi.org/10.1007/s13213-011-0406-3

Blasa M, Angelino D, Gennari L et al (2011) The cellular antioxidant activity in red blood cells (CAA-RBC): a new approach to bioavailability and synergy of phytochemicals and botanical extracts. Food Chem 125(2):685–691. https://doi.org/10.1016/j.foodchem.2010.09.065

Cano-Campos MC, Díaz-Camacho SP, Uribe-Beltrán MJ et al (2011) Bio-guided fractionation of the antimutagenic activity of methanolic extract from the fruit of *Randia echinocarpa* (Sessé et Mociño) against 1-nitropyrene. Food Res Int 44(9):3087–3093. https://doi.org/10.1016/j.foodres.2011.08.006

Costa EM, Silva S, Tavaria FK et al (2017) Insights into chitosan antibiofilm activity against methicillin-resistant *Staphylococcus aureus*. J Appl Microbiol 122(6):1547–1557. https://doi.org/10.1111/jam.13457

Cuevas-Juárez E, Yuriar-Arredondo KY, Pío-León JF et al (2014) Antioxidant and α-glucosidase inhibitory properties of soluble melanins from the fruits of *Vitex mollis* Kunth, *Randia echinocarpa* Sessé et Mociño and *Crescentia alata* Kunth. J Funct Foods 9:78–88. https://doi.org/10.1016/j.jff.2014.04.016

Cuzzubbo S, Carpentier AF (2021) Applications of melanin and melanin-like nanoparticles in cancer therapy: a review of recent advances. Cancers (basel). https://doi.org/10.3390/cancers13061463

de Cassia Ribeiro Goncalves R, Rezende Kitagawa R, Aparecida Varanda E et al (2016) Effect of biotransformation by liver S9 enzymes on the mutagenicity and cytotoxicity of melanin extracted from *Aspergillus nidulans*. Pharm Biol 54(6):1014–1021. https://doi.org/10.3109/13880209.2015.1091846

Dolatabadi S, Moghadam HN, Mahdavi-Ourtakand M (2018) Evaluating the anti-biofilm and antibacterial effects of *Juglans regia* L. extracts against clinical isolates of *Pseudomonas aeruginosa*. Microb Pathog 118:285–289. https://doi.org/10.1016/j.micpath.2018.03.055

El-Abd AO, Bayomi SM, El-Damasy AK et al (2022) Synthesis and molecular docking study of new thiazole derivatives as potential

tubulin polymerization inhibitors. ACS Omega 7(37):33599–33613. https://doi.org/10.1021/acsomega.2c05077

El-Naggar NE, Saber WIA (2022) Natural melanin: current trends, and future approaches, with especial reference to microbial source. Polym. https://doi.org/10.3390/polym14071339

El-Naggar NE-A, El-Ewasy SM (2017) Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. Sci Rep 7(1):42129. https://doi.org/10.1038/srep42129

ElObeid AS, Kamal-Eldin A, Abdelhalim MAK et al (2017) Pharmacological properties of melanin and its function in health. Basic Clin Pharmacol Toxicol 120(6):515–522. https://doi.org/10.1111/bcpt.12748

El-Obeid A, Tahir EL, KEH and Haseeb AM, (2016) Anti-inflammatory effects of *Nigella sativa* L. melanin. World J Pharm Res 5(2):155–161

El-Obeid A, Yahya W, Almuzzaini B et al (2021) Herbal melanin induces interleukin-1β secretion and production by human THP-1 monocytes via Toll-like receptor 2 and p38 MAPK activation. Exp Ther Med. https://doi.org/10.3892/etm.2021.10515

Fernandes JB, Vargas VM (2003) Mutagenic and antimutagenic potential of the medicinal plants *M. laevigata* and *C. xanthocarpa*. Phytother Res 17(3):269–273. https://doi.org/10.1002/ptr.1058

Ferraz AR, Pacheco R, Vaz PD et al (2021) Melanin: Production from cheese bacteria, chemical characterization, and biological activities. Int J Environ Res Public Health. https://doi.org/10.3390/ijerph182010562

Fu X, Xie M, Lu M et al (2022) Characterization of the physicochemical properties, antioxidant activity, and antiproliferative activity of natural melanin from *S. reiliana*. Sci Rep 12(1):2110. https://doi.org/10.1038/s41598-022-05676-z

Gil-Avilés MdR, Montes-Avila J, Díaz-Camacho SP et al (2019) Soluble melanins of the Randia echinocarpa fruit: structural characteristics and toxicity. J Food Biochem 43(12):e13077. https://doi.org/10.1111/jfbc.13077

Glagoleva AY, Shoeva OY, Khlestkina EK (2020) Melanin pigment in plants: current knowledge and future perspectives. Front Plant Sci 11:770. https://doi.org/10.3389/fpls.2020.00770

Goyal M, Baranwal M, Pandey SK et al (2019) Hetero-polysaccharides secreted from *Dunaliella salina* exhibit immunomodulatory activity against peripheral blood mononuclear cells and RAW 264.7 macrophages. Indian J Microbiol 59(4):428–435. https://doi.org/10.1007/s12088-019-00818-w

Hosseini M, Shapouri Moghaddam A, Derakhshan S et al (2020) Correlation between biofilm formation and antibiotic resistance in MRSA and MSSA isolated from clinical samples in Iran: a systematic review and meta-analysis. Microb Drug Resist 26(9):1071–1080. https://doi.org/10.1089/mdr.2020.0001

Hou R, Liu X, Wu X et al (2021) Therapeutic effect of natural melanin from edible fungus *Auricularia auricula* on alcohol-induced liver damage in vitro and in vivo. Food Sci Hum Wellness 10(4):514–522. https://doi.org/10.1016/j.fshw.2021.04.014

Huang S, Pan Y, Gan D et al (2011) Antioxidant activities and UV-protective properties of melanin from the berry of *Cinnamomum burmannii* and *Osmanthus fragrans*. Med Chem Res 20(4):475–481. https://doi.org/10.1007/s00044-010-9341-2

Hung YC, Sava VM, Blagodarsky VA et al (2003) Protection of tea melanin on hydrazine-induced liver injury. Life Sci 72(9):1061–1071. https://doi.org/10.1016/s0024-3205(02)02348-2

Jenny M, Klieber M, Zaknun D et al (2011) *In vitro* testing for anti-inflammatory properties of compounds employing peripheral blood mononuclear cells freshly isolated from healthy donors. Inflamm Res 60(2):127–135. https://doi.org/10.1007/s00011-010-0244-y

Jong-Kyu K, Sun-Mee p, (1995) Novel antimutagenic pigment produced by *Bacillus licheniformis* SSA3. J Microbiol Biotechnol 5(1):48–50

Kado NY, Langley D, Eisenstadt E (1983) A simple modification of the Salmonella liquid-incubation assay. Increased sensitivity for detecting mutagens in human urine. Mutat Res 121(1):25–32. https://doi.org/10.1016/0165-7992(83)90082-9

Kamarudheen N, Naushad T, Rao KVB (2019) Biosynthesis, characterization and antagonistic applications of extracellular melanin pigment from marine *Nocardiopsis* sps. Indian J Pharm Educ Res 53(2s):s112–s120. https://doi.org/10.5530/ijper.53.2s.55

Kannappan A, Gowrishankar S, Srinivasan R et al (2017) Antibiofilm activity of *Vetiveria zizanioides* root extract against methicillin-resistant *Staphylococcus aureus*. Microb Pathog 110:313–324. https://doi.org/10.1016/j.micpath.2017.07.016

Kaplan JB (2011) Antibiotic-induced biofilm formation. Int J Artif Organs 34(9):737–751. https://doi.org/10.5301/ijao.5000027

Kiran GS, Jackson SA, Priyadharsini S et al (2017) Synthesis of Nm-PHB (nanomelanin-polyhydroxy butyrate) nanocomposite film and its protective effect against biofilm-forming multi drug resistant *Staphylococcus aureus*. Sci Rep 7(1):9167. https://doi.org/10.1038/s41598-017-08816-y

Kurian NK, Bhat SG (2018) Food, cosmetic and biological applications of characterized DOPA-melanin from *Vibrio alginolyticus* strain BTKKS3. Appl Biol Chem 61(2):163–171. https://doi.org/10.1007/s13765-018-0343-y

Kurian NK, Nair HP, Bhat SG (2015) Evaluation of anti-inflammatory property of melanin from marine *Bacillus* sp BTCZ31. Asian J Pharm Clin Res 8(3):251–255

Kurian NK and Bhat SG (2017) Photoprotection and anti-Inflammatory properties of non–cytotoxic melanin from marine isolate *Providencia rettgeri* strain. Biosci Biotechnol Res Asia 14(4):1475–1484. https://doi.org/10.13005/bbra/2594

Kurian NK, M L, Nair HP et al. (2015) Melanins with anti-biofilm and anti-inflammatory activities from marine *Pseudomonas stutzeri* BTCZ10. Proced India Int Sci Festiv Young Scientists' Meet. https://doi.org/10.13140/RG.2.1.1806.2163.

Laxmi M, Kurian NK, Smitha S et al (2016) Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. Indian J Biotechnol 15:392–399

Leshem T, Schnall BS, Azrad M et al (2022) Incidence of biofilm formation among MRSA and MSSA clinical isolates from hospitalized patients in Israel. J Appl Microbiol 133(2):922–929. https://doi.org/10.1111/jam.15612

Li Z, Heng H, Qin Q et al (2022) Physicochemical properties, molecular structure, antioxidant activity, and biological function of extracellular melanin from *Ascosphaera apis*. J Zhejiang Univ Sci B 23(5):365–381. https://doi.org/10.1631/jzus.B2100718

Liu X, Hou R, Wang D et al (2019) Comprehensive utilization of edible mushroom *Auricularia auricula* waste residue-extraction, physicochemical properties of melanin and its antioxidant activity. Food Sci Nutr 7(11):3774–3783. https://doi.org/10.1002/fsn3.1239

Liu YM, Ma WS, Wei YX et al (2020) Photothermal effect-based cytotoxic ability of melanin from *Mytilus edulis* shells to heal wounds infected with drug-resistant bacteria *in vivo*. Biomed Environ Sci 33(7):471–483. https://doi.org/10.3967/bes2020.052

Liu S, Youngchim S, Zamith-Miranda D et al (2021) Fungal melanin and the mammalian immune system. J Fungi. https://doi.org/10.3390/jof7040264

Maheshwari S, Kumar V, Bhadauria G et al (2022) Immunomodulatory potential of phytochemicals and other bioactive compounds of

fruits: a review. Food Front 3(2):221–238. https://doi.org/10.1002/fft2.129

Mahmoudi H, Pourhajibagher M, Chiniforush N et al. (2019) Biofilm formation and antibiotic resistance in meticillin-resistant and meticillin-sensitive *Staphylococcus aureus* isolated from burns. J Wound Care 28(2):66–73. https://doi.org/10.12968/jowc.2019.28.2.66.

Makhafola TJ, Elgorashi EE, McGaw LJ et al (2016) The correlation between antimutagenic activity and total phenolic content of extracts of 31 plant species with high antioxidant activity. BMC Complement Alt Med 16(1):490. https://doi.org/10.1186/s12906-016-1437-x

Malich G, Markovic B, Winder C (1997) The sensitivity and specificity of the MTS tetrazolium assay for detecting the *in vitro* cytotoxicity of 20 chemicals using human cell lines. Toxicol 124(3):179–192. https://doi.org/10.1016/s0300-483x(97)00151-0

Marcovici I, Coricovac D, Pinzaru I et al (2022) Melanin and melanin-functionalized nanoparticles as promising tools in cancer research—a review. Cancers. https://doi.org/10.3390/cancers14071838

Maron DM, Ames BN (1983) Revised methods for the *Salmonella* mutagenicity test. Mutat Res 113(3–4):173–215. https://doi.org/10.1016/0165-1161(83)90010-9

Mohammadi M, Masoumipour F, Hassanshahian M et al (2019) Study the antibacterial and antibiofilm activity of *Carum copticum* against antibiotic-resistant bacteria in planktonic and biofilm forms. Microb Pathog 129:99–105. https://doi.org/10.1016/j.micpath.2019.02.002

Montes-Avila J, Ojeda-Ayala M, López-Angulo G et al (2018) Physicochemical properties and biological activities of melanins from the black-edible fruits *Vitex mollis* and *Randia echinocarpa*. J Food Meas Charact 12(3):1972–1980. https://doi.org/10.1007/s11694-018-9812-6

Montes-Avila J, López-Angulo G, Duarte-de-la-Peña G et al (2022) Antioxidant, antibacterial, and antiparasitary activities of green nanoparticles synthesized using water-soluble melanins of fruits. BioNanoSci 12(1):228–240. https://doi.org/10.1007/s12668-022-00940-y

Moreira-Rosa R, Melecchi MI, da Costa HR et al (2006) Antioxidant and antimutagenic properties of Hibiscus tiliaceus L methanolic extract. J Agric Food Chem 54(19):7324–7330. https://doi.org/10.1021/jf061407b

Oh JJ, Kim JY, Son SH et al (2021) Fungal melanin as a biocompatible broad-spectrum sunscreen with high antioxidant activity. RSC Adv 11(32):19682–19689. https://doi.org/10.1039/d1ra02583j

Ojeda-Ayala M, Gaxiola-Camacho SM and Delgado-Vargas F (2022) Phytochemical composition and biological activities of the plantas of the genus *Randia*. Bot Sci 100(4):779–796. https://doi.org/10.17129/botsci.3004

Petrosyan T, Hovsepyan A, Avetisyan S et al (2019) *In-vitro* effects of bacterial melanin in macrophage "RAW 264.7" cell culture. Adv Neuroimmune Biol 7:199–206. https://doi.org/10.3233/NIB-190162

Piechota M, Kot B, Frankowska-Maciejewska A et al (2018) Biofilm formation by methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* strains from hospitalized patients in Poland. Biomed Res Int 2018:1–7. https://doi.org/10.1155/2018/4657396

Pío-León JF, Montes-Avila J, López-Angulo G et al (2018) Melanins of *Vitex mollis* fruit with differences in water-solubility show high inhibition of carbohydrate digestive enzymes and antioxidant activity. J Food Biochem. https://doi.org/10.1111/jfbc.12509

Qais FA, Khan MS, Ahmad I (2019) Broad-spectrum quorum sensing and biofilm inhibition by green tea against gram-negative pathogenic bacteria: deciphering the role of phytocompounds through molecular modelling. Microb Pathog 126:379–392. https://doi.org/10.1016/j.micpath.2018.11.030

Santos-Cervantes ME, Ibarra-Zazueta ME, Loarca-Piña G et al (2007) Antioxidant and antimutagenic activites of *Randia echinocarpa* fruit. Plant Foods Hum Nutr 62:71–77. https://doi.org/10.1007/s11130-007-0044-x

Singh S, Datta S, Narayanan KB et al (2021) Bacterial exo-polysaccharides in biofilms: role in antimicrobial resistance and treatments. J Gen Eng Biotechnol. https://doi.org/10.1186/s43141-021-00242-y

Sloczynska K, Powroznik B, Pekala E et al (2014) Antimutagenic compounds and their possible mechanisms of action. J Appl Genet 55(2):273–285. https://doi.org/10.1007/s13353-014-0198-9

Surendirakumar K, Pandey RR, Muthukumar T et al (2022) Characterization and biological activities of melanin pigment from root endophytic fungus, *Phoma* sp. RDSE17. Arch Microbiol 204(3):171. https://doi.org/10.1007/s00203-022-02788-y

Tajima K, Yamanaka D, Ki I et al (2019) Solubilized melanin suppresses macrophage function. FEBS Open Bio 9(4):791–800. https://doi.org/10.1002/2211-5463.12615

Venkatalakshmi P, Vadivel V, Brindha P (2016) Role of phytochemicals as immunomodulatory agents: a review. Int J Green Pharm 10(1):1–18

Venkatesh S, Ravi L, Rajendran A (2018) Role of oral mucosal melanin-from birth to adulthood in primary preference. Antibacterial, antifungal and antibiofilm but not anti-inflammatory activity. Int J Adv Res 4:1245–1258

Wang L, Li Y, Li Y (2019) Metal ions driven production, characterization and bioactivity of extracellular melanin from *Streptomyces* sp. ZL-24. Int J Biol Macromol 123:521–530. https://doi.org/10.1016/j.ijbiomac.2018.11.061

Wold CW, Gerwick WH, Wangensteen H et al (2020) Bioactive triterpenoids and water-soluble melanin from *Inonotus obliquus* (Chaga) with immunomodulatory activity. J Funct Foods. https://doi.org/10.1016/j.jff.2020.104025

Xu L, Li J, Chang M et al (2020) Comparison of physicochemical and biochemical properties of natural and arginine-modified melanin from medicinal mushroom *Ganoderma lucidum*. J Basic Microbiol 60(11–12):1014–1028. https://doi.org/10.1002/jobm.202000430

Ye Z, Lu Y, Zong S et al (2019) Structure, molecular modification and anti-tumor activity of melanin from *Lachnum singerianum*. Process Biochem 76:203–212. https://doi.org/10.1016/j.procbio.2018.09.007

Yesmin S, Paul A, Naz T et al (2020) Membrane stabilization as a mechanism of the anti-inflammatory activity of ethanolic root extract of Choi (*Piper chaba*). Clin Phytoscience 6(1):59. https://doi.org/10.1186/s40816-020-00207-7