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Mechanisms of antioxidants in the treatment of vitiligo *in vivo*: a systematic review

Mecanismos de antioxidantes no tratamento do vitiligo *in vivo*: uma revisão sistemática

Mecanismos de los antioxidantes en el tratamiento del vitíligo *in vivo*: una revisión sistemática

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Abstract
Vitiligo is characterized by skin discoloration in different body regions, and its prevalence in the world population can reach 1%. Its cause is still undefined but possibly related to biochemical, environmental, immunological, and genetic events. In addition, individuals with vitiligo present electrolyte disturbances in the melanocytes, and it has been one of the targets of studies for treating this pathology. This systematic review aimed to gather and evaluate *in vivo* studies that described the mechanism of action of antioxidants in vitiligo. PubMed, Web of Science, Embase, Science Direct, LILACS, Open Grey and Google Scholar were used as research sources. The search included all articles up to October 16, 2021, with no start date restrictions. The search yielded 390 articles, and 7 of these were selected according to the inclusion criteria. The compounds studied in the articles selected for this review were curcumin, Cold Atmospheric Plasma, butin, galangin, 2',3,4,4'-tetrahydrochalcone and its derivatives, 6-benzylaminopurine, piperine, chrysin, kaempferol, scopoletin, vitamin D3, caffeic acid, and luteolin. The results showed that the main mechanisms of action of antioxidants in the treatment of vitiligo are the increase in tyrosinase activity by increasing the synthesis of TYR and TRP-1, the positive regulation of the number of epidermal cells, the redox balance, activation and regulation of MITF, and malondialdehyde and cholinesterase/acetylcholinesterase content. Thus, these pathways can be considered targets for evaluating different antioxidant compounds to act in treating vitiligo.
Keywords: Vitiligo; Antioxidants; Molecular mechanisms of pharmacological action; Biological assay; Systematic review.

Resumo
O vitiligo é caracterizado pela descoloração da pele em diferentes regiões do corpo, e sua prevalência na população mundial pode chegar a 1%. Sua causa ainda não está totalmente elucidada, mas possivelmente está relacionada a eventos bioquímicos, ambientais, imunológicos e genéticos. Além disso, indivíduos com vitiligo apresentam distúrbios eletrolíticos em seus melanócitos, e este tem sido um dos alvos de estudos para tratamento desta patologia. Esta revisão sistemática teve como objetivo reunir e avaliar estudos *in vivo* que descreveram o mecanismo de ação de antioxidantes no vitiligo. As bases de dados PubMed, Web of Science, Embase, Science Direct, LILACS, Open Grey e Google Scholar foram utilizadas como fonte de pesquisa. A busca incluiu todos os artigos até 16 de outubro de 2021, sem restrições de data de início. A pesquisa resultou em 390 artigos, e 7 desses foram selecionados de acordo com os critérios de inclusão. Os compostos estudados nos artigos selecionados foram curcumina, *Cold Atmospheric Plasma*, butina, galangina, 2',3,4,4'-tetrahidrochalcona e seus derivados, 6-benzilaminopurina, piperina, crisina, kaempferol, escopoletina, vitamina D3, ácido cafeico e luteolina. Os resultados demonstraram que os principais mecanismos de ação dos antioxidantes no tratamento do vitiligo são o aumento da atividade da tirosinase pelo aumento da síntese de

TYR e TRP-1, a regulação positiva do número de células epidérmicas, o balanço redox, ativação e regulação do MITF, e conteúdo de malondialdeído e colinesterase/acetilcolinesterase. Assim, essas vias podem ser consideradas alvos para avaliação de diferentes compostos antioxidantes para atuar no tratamento do vitiligo.
Palavras-chave: Vitiligo; Antioxidantes; Mecanismos moleculares de ação farmacológica; Bioensaio; Revisão sistemática.

Resumen
El vitíligo se caracteriza por la decoloración de la piel en diferentes regiones del cuerpo, y su prevalencia en la población mundial puede llegar al 1%. Su causa aún no está todo aclarada, pero posiblemente esté relacionada con eventos bioquímicos, ambientales, inmunológicos y genéticos. Además, las personas con vitíligo presentan alteraciones electrolíticas en sus melanocitos, y este ha sido uno de los objetivos de estudios para el tratamiento de esta patología. Esta revisión sistemática tuvo como objetivo recopilar y evaluar estudios *in vivo* que describieran el mecanismo de acción de los antioxidantes en el vitíligo. Se utilizaron como fuentes de investigación las bases de datos PubMed, Web of Science, Embase, Science Direct, LILACS, Open Grey y Google Scholar. La búsqueda incluyó todos los artículos hasta el 16 de octubre de 2021. La búsqueda resultó en 390 artículos, y 7 de estos fueron seleccionados de acuerdo con los criterios de inclusión. Los compuestos estudiados en los artículos seleccionados fueron curcumina, plasma atmosférico frío, butina, galangina, 2',3,4,4'-tetrahidrocalcona y sus derivados, 6-bencilaminopurina, piperina, crisina, kaempferol, escopoletina, vitamina D3, ácido cafeico y luteolina. Los resultados mostraron que los principales mecanismos de acción de los antioxidantes en el tratamiento del vitíligo son el aumento de la actividad tirosinasa al incrementar la síntesis de TYR y TRP-1, regulación positiva del número de células epidérmicas, balance redox, activación y regulación. de MITF, y contenido de malondialdehído y colinesterasa/acetilcolinesterasa. Estas vías pueden considerarse dianas para la evaluación de diferentes compuestos antioxidantes para actuar en el tratamiento del vitíligo.
Palabras clave: Vitíligo; Antioxidantes; Mecanismos moleculares de acción farmacológica; Bioensayo; Revisión sistemática.

1. Introduction

Vitiligo is a chronic hypopigmenting pathology that does not cause systemic damage. Its main feature is the loss of melanocytes resulting in white macules on the skin without signs of inflammation or scales. Its prevalence is 0.5 to 1% of the world population (Gowda, et al., 2020; Rashighi & Harris, 2017; Seneschal, et al., 2021). Despite not being a potentially fatal disease, it deserves attention because it can be associated with psychological issues such as low self-esteem, insecurity, anxiety, and depression (Bergqvist & Ezzedine, 2020; Ezzedine, et al., 2015; Meneghin, 2021; Salzes, et al., 2015).

The skin is the largest organ in the body and accommodates the cells responsible for producing and storing melanin - a fundamental pigment in photoprotection (Cordero & Casadevall, 1997; Nichols & Katiyar, 2010). Melanocytes constitute 1% of the skin cells, and one of its organelles is the melanosome, which is responsible for synthesizing melanin. Each melanocyte is interconnected with 30 to 40 keratinocytes through dendrites, so melanosomes containing melanin are transported from melanocytes to keratinocytes and stored (Rzepka, et al., 2016; Videira, et al., 2013).

In parallel with the autoimmunity theory, the most supported etiology for vitiligo (Kemp, et al., 2001; Rezaei, et al., 2007; Van Den Wijngaard, et al., 2001), oxidative stress is also a significant risk factor for vitiligo (Speeckaert, et al., 2018; Sun, et al., 2020; Wang, et al., 2019), because reactive oxygen species cause a senescent phenotype in cells that can trigger the autoimmunity (Picardo, et al., 2015). Reactive oxygen species (ROS) are generated daily during various cellular processes or by exogenous ROS-generating stimuli such as pollution, atmospheric gases, trauma, stress, severe infection, and ultraviolet (UV) radiation (Turrens, 2003; Xie, et al., 2016). A potential mechanism for preventing the onset of pathologies related to oxidative stress is using substances with antioxidant properties (Speeckaert, et al., 2018; Zouboulis & Makrantonaki, 2011).

The purpose of this review was to search and describe the effects of the antioxidant compounds in the vitiligo treatment *in vivo*, aiming to gather the available data on the possible action mechanisms of *in vivo* antioxidants for vitiligo treatment.

2. Methodology

2.1 Protocol and registration

This systematic review was prepared following the recommendations of PRISMA 2020 (Preferred Reporting Items for Systematic Reviews and Meta-Analysis). The review protocol was registered in the PROSPERO (International Prospective Systematic Reviews Registry) under registration number 300518.

The methodology employed in this systematic review followed the recommendations of De Luca Canto (2020).

2.2 Eligibility criteria

The research question this review aimed to answer was "What are the mechanisms of action of the antioxidants described in biological assays for the treatment of vitiligo?" The PICOS acronymous was used to development of the research question and to elaborate the search strategy (De Canto Luca, 2020).

The PICOS acronymous was defined as: *(P)* population: zebra-fish (*Danio rerio*) or murine models; *(I)* intervention: treatment with antioxidants; *(C)* comparison: animals not treated; *(O)* outcomes: Primary outcome: mechanism of action of the antioxidants in vitiligo treatment. Secondary outcomes: induction of melanogenesis, the viability of melanocytes, and expression of genes related to melanogenesis (TYR, TRP-1, TRP-2, MAPK, p38 MAPK, PKA, NRF2, ERK, MITF, and KIT); (S) study design: *in vivo* assays.

2.3 Inclusion criteria

Articles that evaluated the induction of melanogenesis (melanin production, melanocyte viability, tyrosinase gene expression, tyrosinase activity) and activation of mechanisms such as regulation of MITF transcriptional activity by MAPK pathways (ERK, JNK, and p28) and regulation of TYR, TRP-1, and TRP-2 expression, as result of *in vivo* treatment with antioxidants.

2.4 Exclusion Criteria

Studies were excluded for the following reasons: studies that used compounds to treat vitiligo that did not have antioxidant activity, *in vivo* studies in other animal models than murine or zebrafish, *in vitro* studies only, human studies, *ex vivo* studies only, *in silico* studies only and studies without a control group.

2.5 Information Sources and Search Strategy

The search was performed in PubMed, Web of Science, Embase, ScienceDirect, and LILACS databases and grey literature by Open Grey and Google Scholar. An individual search strategy was elaborated for each database, comprising terms and MESH terms related to vitiligo, melanogenesis, antioxidants, and possible signaling pathways evolved in melanogenesis and antioxidant activity. The individual search used in each database is detailed in Supplemental Table 1. The searches included all articles up to October 16, 2021, with no restrictions on the initial publication date or language. In addition, a manual search was carried out in the reference list of selected articles in search of possible relevant studies.

2.6 Selection of studies

The articles were selected from the databases and exported to the Rayyan tool in the first step. Duplicate articles were excluded, and the titles and abstracts of the remaining articles were blindly examined by Paloma de Jesus (PJ) and Manuel Mera (MM). Based on the inclusion and exclusion criteria, the articles were selected. In the second stage, a complete reading

of the articles was performed using the same criteria to confirm the eligibility or not of the articles selected initially. At this stage, a discussion with the third author, Maria de Fátima (MF), was necessary for a mutual agreement.

2.7 Data collection and analysis process

Information collected included: authors, year, country, intervention, study design, sample size, sex, age, weight, treatment dose, trials, mechanisms, and main findings. The first author (PJ) collected the main information from the selected articles and structured the summary table. The second (MM) and third (MF) reviewers verified the information and the veracity of the data, and the divergences were discussed until reaching a consensus.

Due to the absence of quantitative data in some of the reported results, only qualitative analysis was performed. The measures of the effect of the antioxidants in the primary or secondary outcomes were summarized by dichotomous categorical variables (yes/no) (Brasil, 2012).

2.8 Risk of bias and quality assessment

To assess the risk of bias in the studies included in this review, the RoB tools from SYRCLE and CAMARADES were adopted (Hooijmans, et al., 2014; Macleod, et al., 2004). The first and second reviewers rated the 10-item articles as Yes, low risk of bias; No, high risk of bias; or Unclear, moderate risk of bias. Items evaluated included: peer review, temperature control, random allocation to treatment or control, blind induction of vitiligo, blinded outcome evaluation, use of an anesthetic without significant intrinsic protective activity to vitiligo, animal model characteristics, sample, compliance with welfare regulations, and declaration of potential conflict of interest. Differences were resolved with the third author.

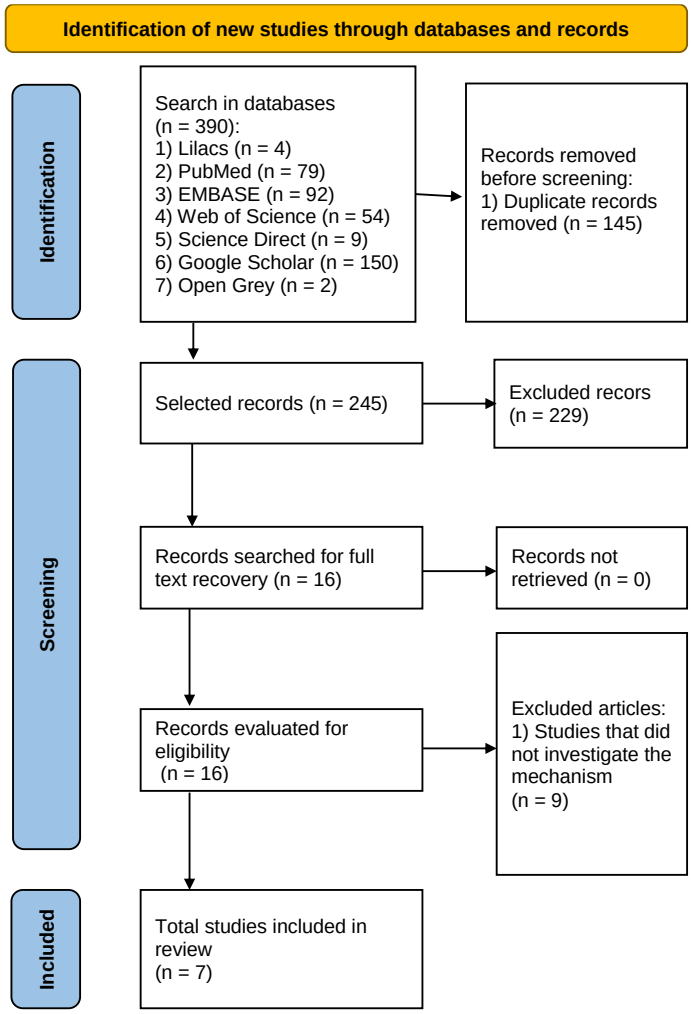
2.9 Summary measures

For this SR, the primary outcome evaluated in the studies was the effect of antioxidants in the treatment of vitiligo, evaluated through the restoration of redox balance; regulation of MITF, TYR, TRP-1, TRP-2, tyrosine kinase receptor (kit), and dopachrome tautomerase (dct) expression; regulation of iNOS expression; regulation of NRF2 expression; regulation of the amount of melanin-containing cells and regulation of melanin synthesis. The secondary outcome was the induction of melanogenesis, melanocyte viability, and expression of genes related to melanogenesis (TYR, TRP-1, TRP-2, MAPK, p38 MAPK, PKA, NRF2, ERK, MITF, and kit). The format of data extraction was dichotomous (yes/no).

3. Results

The initial search identified 390 articles from the seven databases. After removing the duplicate articles, 245 articles remained for screening. Of these, 229 were excluded after reading the titles and abstracts, resulting in 16 articles being thoroughly evaluated. After a comprehensive analysis, 9 studies were excluded, and 7 were included in the qualitative synthesis. No additional articles were identified in the reference lists of selected studies. A detailed flowchart with the process of identification, exclusion, and inclusion of studies is represented in Figure 1.

Figure 1 - Flowchart of the literature search and selection process.



Flowchart adapted from: PRISMA Statement 2020: an up-to-date guide to publishing systematic reviews. Page, et al., (2021).

Study characteristics

The selected articles were published between 2014 and 2021, three from 2021(Abuduaini, et al., 2021; Lai, et al., 2021; Zhai, et al., 2021). The language of all articles was English, and the country of publication of all articles was China. Antioxidants evaluated in the studies joined in this review comprised curcumin tablet (CWT) (Abuduaini, et al., 2021); 6-benzylaminopurine, piperine (pip), chrysin (chr), kaempferol (kaem), scopoletin (sco), vitamin D3 (VitD3), (Heriniaina, et al., 2018); RY3-a, CY3-a-1, CY3-a-15, RY3-b, RY3-c (Zhong, et al., 2019); cold atmospheric plasma (CAP) (Zhai, et al., 2021); butin (Huo, et al., 2017; Lai, et al., 2021); caffeic acid and luteolin (Lai, et al., 2021), and galangin (Huo, et al., 2014). The results of all published works on these antioxidants suggest that at least one of the antioxidants investigated in each article is promising for treating vitiligo.

The studies included in this review used mice (n = 5) or zebrafish (n = 3) as the *in vivo* models, and one of the articles (Zhong, et al., 2019) used both these *in vivo* models. For articles that carried out *in vitro* investigation, the B16F10 cell line was used. Three articles that included *in vitro* studies evaluated cell viability by MTT (Abuduaini, et al., 2021; Heriniaina, et al., 2018; Lai, et al., 2021), and one evaluated cell viability by flow cytometry (Zhong, et al., 2019).

All articles that reported *in vivo* studies with mice used the inbred C57BL/6 strain. Four studies induced vitiligo in mice through hydroquinone treatment (Abuduaini, et al., 2021; Huo, et al., 2014; Huo, et al., 2017; Zhong, et al., 2019), and one of them induced vitiligo by treatment with monobenzene (Zhai, et al., 2021). In the zebrafish model, vitiligo was induced by PTU in all three reports (Heriniaina, et al., 2018; Lai, et al., 2021; Zhong, et al., 2019). Three of the compounds studied were administered orally in the mouse models, CWT, butin, and galangin (Abuduaini, et al., 2021; Huo, et al., 2014, 2017), and six compounds, CAP, RY3-a, CY3-a- 1, CY3-a-15, RY3-c, and RY3-b were administered topically (Xia, et al., 2019; Zhong, et al., 2019). In the zebrafish model, butin, 6-bap, pip, chr, Kaem, sco, and VitD3 were administered by enriching the fish incubation medium with the respective compounds (Heriniaina, et al., 2018; Huo, et al., 2017).

It was a condition for inclusion in the systematic review that the articles had an *in vivo* study with zebrafish or mice. A constant feature in the selected studies is that, except for Huo, et al., 2017, they all brought images showing visible results of the effects in mice and zebrafish. It was possible to observe an increase in fur coloration in mice and the head and back in zebrafish embryos. Among the selected articles, all that mentioned the study pattern did so in a randomized manner, and all the mouse studies cited sample size, sex, age, intervention doses, and respective weight margins, except Zhong, et al., 2019, which did not report the weight of the animals. The zebrafish studies did not mention a study pattern. The descriptive characteristics of each article were compiled in Table 1.

Table 1 - Summary of descriptive characteristics of articles included in the review (n = 7).

First author, year, country	Intervention	Study design	Sample size, sex, age, weight	Dose	Assays to investigate the mechanisms	Mechanism	Main conclusions
Abuduaini, A., 2021, China	CWT - oral administration	Mice C57BL/6 Randomized controlled trial	Total of 72 mice C57BL/6: 12 animals per group, male and female, 5-6 weeks, 20 ± 2g	**Control** White group, untreated: treatment with purified water, 0 mg/kg CWT or BC **Treatment** Model disease group: 0 mg CWT or BC/kg; Positive control of the BC group: BC 720 mg/kg; CWT-L group: 100 mg CWT/kg; CWT-M Group: 200 mg CWT/Kg; CWT-H Group: 400 mg/Kg	1. Histological analysis of mouse skin 2. Mouse serum analysis 3. Analysis of TYR, MDA, MAO, and AChE in mouse skin tissues 4. Analysis of the expression of TYR, TRP-1, and TRP-2 proteins in mouse skin	1. The number of melanin-containing hair follicles in treated areas was increased at all CWT dosages. Basal melanocytes and melanin-containing epidermal cells increased significantly at 200 and 400 mg/kg CWT concentrations. 2. TYR levels after treatment were higher than the model group, and the content of MDA was lower. 3. TYR content in the treated groups was visibly increased, and MDA content and MAO levels decreased. Ache levels were decreased, but insignificantly. 4. Treatment effects produced an increase in the mean density of TYR, TRP-1, and TRP-2 levels in tissue in a dose-dependent manner.	Treatment with CWT attenuated hydroquinone-induced vitiligo in mice. It increased the number of melanin-containing cells and the TYR content in mice's blood and skin tissue and decreased the MDA levels in both samples. In addition, it promoted an increase in the expression of melanogenic proteins.
Lai, Y., 2021, China	CAP jet or CAP activated hydrogel - topical application.	Mice C57BL/6 Randomized study	24 female C57BL/6 mice at 4 weeks of age, 13 - 16 g	**Control** No treatment **Treatment** CAP activated hydrogel group: once every other day CAP jet group: weekly 1.5-minute CAP jet placed 10 mm upstream of the mouse skin	1. H_2O_2 and NO assays with a commercial H_2O_2 assay kit (Beyotime Biotechnology, Shanghai, China) and Griess assay kit (Beyotime Biotechnology) to infer NO. 2. PCR - commercial kit (Takara Bio, Kyoto, Japan), TB Green stain (Takara Bio, Beijing, China), and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Waltham,	Down-regulation of iNOS expression and up-regulation of NRF2 expression.	This study demonstrates that CAP jet or CAP-activated hydrogel treatment can improve vitiligo lesions in mice with vitiligo without adverse effects in this first follow-up period. Thus, CAP treatment can effectively and safely alleviate vitiligo by modulating the oxidative stress response.

					MA) 3. Western Blotting		
Huo, S.X., 2017, China	Butin - oral administration	Mice C57BL/6 Randomized study	96 male C57BL/6 mice aged 5 to 6 weeks, 20 ± 2 g	**Control** distilled water and sodium carboxymethyl cellulose (CMC-Na, 0.5%) **Treatment** 8-MOP: 4.25 mg/kg Butin: 0.425, 4.25 and 42.5 mg/kg	1. Pathomorphology 2. Immune analysis 3. Biochemical measures	Increases the expression of TYR and TRP-1. Increases melanin-containing epidermal cells. Decreases CHE activity and MDA content	Treatment with butin increased the expression of TYR and TRP-1, increased the number of melanin-containing epidermal cells, and decreased the activity of CHE and the MDA content, which are the main mechanisms responsible for the recovery of vitiligo.
Huo, S. X., 2014, China	Galangin - oral administration	Mice C57BL/6 Randomized study	60 C57BL mice / 6 males, aged 5 to 6 weeks, 20 ± 2 g	**Control** distilled water and sodium carboxymethyl cellulose (CMC-Na, 0.5%) **Treatment** Galangin: 0.425 and 4.25 mg/kg	1. Histological analysis 2. Spectrophotometry 3. Immunohistochemistry 4. CHE activity and serum MDA content analyzed by kit	After galangin treatment, the hair color darkened, and histological analysis showed that melanin-containing cells increased. Serum analysis showed that the amount of TYR increased, the MDA content decreased, and the CHE activity decreased. Skin analysis also showed increased expression of the TYR protein.	The results of this study showed that galangin improves vitiligo lesions in mice. The mechanisms involve activities related to TYR concentrations, TYR protein expression, CHE activity, and MDA content.
Zhong, H., 2019, China	RY3-a: flavonoid compound 2,3,4,4'-tetrahydrochalcone, and its derivatives: CY3-a-1 – CY3-a-15, RY3-c, and RY3-b – topical administration	1. Zebrafish model. Randomization not mentioned 2. Mice C57BL/6 Randomized study	1. Information not mentioned for the zebrafish model 2. Mice: 48, male, age and weight not mentioned	**<u>Zebrafish</u>** **Control** Water **Treatment** PTU (0.2 mmol/L); RY3-a, RY3-b and RY3-c in concentrations: 1 µmol/L, 10 µmol/L, 20 µmol/L and 40 µmol/L **<u>Mice</u>** **Control** placebo ointment **Treatment** 8-MOP (8.5 µg/day) and RY3-c (10 µg/day) for 40 days	1) Investigated using DCFH-DA probes in zebrafish whether RY3-a, RY3-b, and RY3-c exert antioxidant activity.	Zebrafish model: acts on the MAPK signaling pathway and reverses ROS content. Mouse model: Not mentioned. This study demonstrated that RY3-c has potent antioxidant activity, can repair cell damage induced by excessive oxidative stress, and act on melanogenesis via the MAPK pathway.	RY3-a, CY3-a-1 - CY3-a-15, RY3-b, and RY3-c, RY3-c stood out with higher melanogenic activity among the components studied. This component has potent antioxidant activity and was able to repair cell damage induced by excessive oxidative stress. In addition, RY3-c acted mainly in the p38-MAPK and ERK1/2 pathways and played an essential role in melanin synthesis.

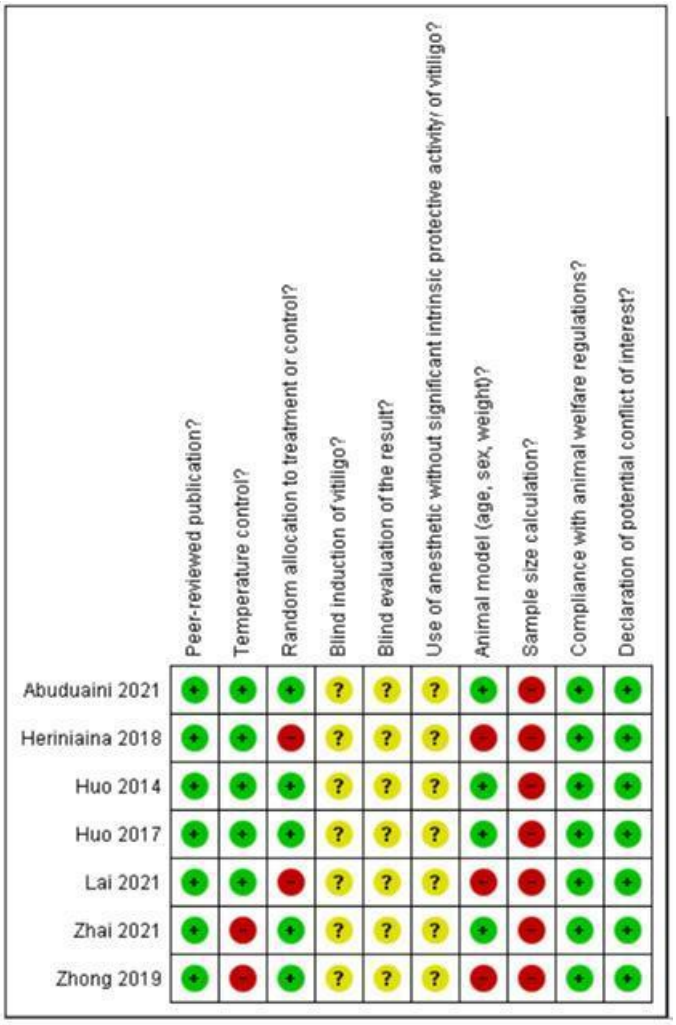
Heriniaina, R.M., 2018, China	6-bap, pip, chr, kaem, sco e VitD3 – administration by incubation	Zebrafish Randomization not mentioned	10 embryos	Effects on melanogenesis: Control: zebrafish embryo medium Treatment: 6 - bap: 20 µmol/L PTU: 0.2 mmol/L Antioxidant activities of 6-bap compared to chr: Control: zebrafish embryo medium Treatment: H_2O_2: 200 µmol/L 6- benzylaminopurine: 30 µmol/L	1. Effect of 6-bap on tyrp1a and mitfa in transgenic zebrafish 2. Antioxidant activities of 6 bap compared to chr (stress induced by H_2O_2, fluorescent probe DCFH-DA).	Upregulation of mitfa, tyr, and tyrp1 protein expression.	The six compounds were shown to have the ability to activate melanogenesis, but 6-bap stood out among them. Despite having low antioxidant power, it can directly increase melanin production.
Lai, Y., 2021, China	Butin, caffeic acid, and luteolin - administration by incubation	Zebrafish Randomization not mentioned	***In situ* hybridization in whole-mount zebrafish embryos:** Each well was filled with 40 embryos. **Antioxidant activities in zebrafish with DCFH-DA fluorescent probe:** Amount not mentioned. **Zebrafish Chemically Induced Inflammation Assay:** Six-well plates with 10–20 tails per well. **Melanogenesis and TYR activity in zebrafish:** Amount not mentioned	**Control** Zebrafish embryo medium **Treatment** butin 0–150 µmol/L, caffeic acid 0–200 µmol/L, luteolin 0–80 µmol/L	1. TYR activity in zebrafish was examined by measuring the rate of oxidation of L-DOPA. 2. Antioxidant activities in zebrafish with DCFH-DA 3. *In situ* hybridization in the mounted zebrafish embryo	It increases the expression of melanogenic genes (tyr, mitfa, kit, and dct), stimulating tyrosinase activity and ROS inhibition.	Butin, caffeic acid, and luteolin promoted proliferation, differentiation, and melanin synthesis of melanocytes when applied independently but were even more efficient combined in a specific ratio (butin: caffeic acid: luteolin = 1:4:10).

Subtitle: CWT: curcumin tablet; TYR: tyrosinase; TRP-1: protein related to tyrosinase 1; TRP-2: protein related to tyrosinase 2; MAPK: mitogen-activated protein kinase; p38 MAPK: mitogen-activated p38 protein kinase; PKA: Protein kinase A; CAP: Cold Atmospheric Plasma; iNOS: inducible nitric oxide synthase; NRF2: erythroid nuclear factor 2; 8-MOP: 8-methoxypsoralen; MDA: malondialdehyde; MAO: monoamine oxidase; CHE: cholinesterase; RY3-b: Butin; RY3-a: 2',3,4,4'-tetrahydrochalcone; RY3-a-1 – RY3-a-15 and RY3-c: RY3-a analogues; PTU: N-phenylthiourea; ROS: reactive oxygen species; ERK: extracellular signal-regulated kinase; 6-bap: benzylaminopurine; pip: piperine; chr: chrysin; Kaem: kaempferol; sco: scopoletin; VitD3: vitamin D3; MITF: melanocyte-inducing transcription factor; dct: L-dopachrome tautomerase; kit: Tyrosine Kinase Receptor. Source: Authors.

Risk of bias

The result of the risk of bias analysis using CAMARADES tool is summarized in Table 2. Criteria related to peer-reviewed publication (item 1), compliance with animal welfare declaration regulations (item 9), and a potential conflict of interest were scored as 'yes' in all articles, showing a low risk of bias. References to blind induction of vitiligo (item 4), blind evaluation of the result (item 5), and use of anesthesia (item 6) were scored as “unclear item”, not being possible to clearly define the quality of the information. Two studies scored 'no' because they lacked data about temperature control (item 2). Three others also scored "no" for not fully describing the characteristics of animal models (item 7), and two for not reporting the random allocation to treatment or control (item 3). The missing information in all selected articles was about the sample size calculation (item 8). Consequently, there was a high risk of bias in these parameters.

Table 2 - Risk of bias in individual studies by the CAMARADES criteria (Macleod, et al., 2004).



Subtitle: +(green), low risk; ?(yellow), unclear; -(red), high risk. Source: Authors.

CAMARADES (Collaborative Approach to Meta-analysis and Review of Experimental Studies in Animals) is a research group founded in 2004 whose focus is to minimize translational flaws in preclinical research. The Rob tool is an adaptation of the Cochrane RoB tool and aspects of bias are employed in animal intervention studies. The tools consist of a set of 10 questions related to selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases. For each of the questions, it is possible to answer “Yes”, when the requested criterion is described, “No”, when the study does not

meet the requested criterion, and "Unclear" when the information is not present or was only partially answered.

The seven selected articles showed an uncertain risk of bias when evaluated with Rob from SYRCLE or CAMARADES tools. The studies did not describe all information about allocation of the animals, randomization, and blinding of studies. These characteristics are fundamental for assessing the quality of studies.

4. Discussion

Vitiligo is a pathology that affects both sexes without distinction, it is not potentially fatal, but due to its main physical characteristic (white macules on the skin), it may be related to psychological comorbidities (Ding, et al., 2021; Salzes C., et al., 2015). Current treatments for vitiligo may include pharmacological, surgical, cosmetic, and phototherapy interventions, but these interventions are still limited, and there is no satisfactory cure (Daniel & Wittal, 2015; Guerra, et al., 2010; Karagaiah, et al., 2020).

Melanin, the natural skin pigment responsible for photoprotection, is synthesized by the complex cascade of melanogenesis. The lack of skin color in the individual with vitiligo is due to the death of melanocytes or the blockage of the melanogenesis pathway. A master regulator of this pathway, MITF, is responsible for upregulating the enzymes tyrosinase (TYR), tyrosine-related protein-1 (TYRP-1), and tyrosine-related protein-2 (TIRP-2) (Hwang, et al., 2019; Niu, et al., 2018; Niu & Aisa, 2017). The tyrosinase enzyme deserves to be highlighted for being multifunctional and crucial in the synthesis of melanin by participating in several steps and limiting the process (Niu & Aisa, 2017). This enzyme is responsible for catalyzing the conversion of L-tyrosine to levodopa (L-DOPA) and L-DOPA to dopaquinone. Dopaquinone is then converted, via proteins TRP-1 and TRP-2, into eumelanin. On the other hand, pheomelanin depends on the conversion of dopaquinone through cysteine or glutathione (Rzepka, et al., 2016; Serre, et al., 2018).

The epidermis of a patient with vitiligo has an oxidative disorder that is not restricted to the injured regions. In this situation, excess H_2O_2 leads to cell apoptosis, which may be the initial triggering event for vitiligo (Glassman, 2011; Schallreuter, et al., 1999). Therefore, a potential mechanism for preventing the onset of pathologies related to oxidative stress is using substances with antioxidant properties (Zhang, et al., 2022).

Thus, this systematic review evaluated the *in vivo* assays effects of different antioxidants on melanogenesis by the following parameters: restoration of redox balance, upregulation of MITF, TYR, TRP-1, TRP-2, kit, and dct expression; upregulation of NRF2; upregulation of MAPK; upregulation of the amount of melanin-containing cells, direct upregulation in melanin synthesis, downregulation of iNOS expression, decreasing of malondialdehyde (MDA) content, and decreasing in the levels of cholinesterase (CHE)/acetylcholinesterase (Ache), as shown in Table 3. The seven studies reported that at least one of the antioxidants investigated in each article noticeably induces melanogenesis.

Table 3 - Relationship between the mechanisms and the respective antioxidants tested.

Mechanism	**Compound evaluated**
Upregulation of TYR expression	CWT Butin Galangin 6-bap Butin:caffeic acid:luteolin (1:4:10)
Upregulation of TRP-1 expression	CWT Butin Galangin 6-bap
Upregulation of the epidermal cells	CWT Butin Galangin
Restoration of redox balance	CWT RY3-c Butin:caffeic acid:luteolin (1:4:10)
Upregulation of mitfa expression	6-bap Butin:caffeic acid:luteolin (1:4:10)
Decreasing malondialdehyde (MDA) and cholinesterase (CHE) / Acetylcholinesterase (AChE) contents	CWT Butin Galangin
Upregulation of TRP-2 expression	CWT
Upregulation of kit and dct expression	Butin:caffeic acid:luteolin (1:4:10)
Upregulation of NRF2	CAP
MAPK upregulation	RY3-c
Direct upregulation of melanin synthesis	6-bap
Downregulation of iNOS expression	CAP
Downregulation of MAO	CWT

Subtitle: CWT, curcumin tablet; 6-bap, benzylaminopurine; RY3-c, 2',3,4,4'-tetrahydrochalcone analogue; CAP, Cold Atmospheric Plasma. Source: Authors.

As observed in Table 3, curcumin tablets, followed by butin and galangin, showed effect on the higher number of melanogenesis parameters evaluated.

The use of antioxidants as a therapeutic strategy has been increasingly studied (Jung, et al., 2018). In this systematic review, 12 investigated compounds were from natural origin (CWT, 6-bap, pip, chr, kaem, sco, 2',3,4,4'-tetrahydrochalcone – RY3-a, butin, galangin, caffeic acid. luteolin and VitD3) and the CAP jet is the only exception. Malondialdehyde (MDA), a lipid peroxidation product, is an important biomarker in evaluating oxidative stress and was one of the parameters evaluated in the studies. This compound is often found at high levels in pathologies related to oxidative stress (Arican & Kurutas, 2008; Yildirim, et al., 2004). Another possible biochemical measurement to assess oxidative stress is the mensuration of monoamine oxidase (MAO) and cholinesterase (CHE). MAO is a monoamine-degrading enzyme present in animals, and its increase favors toxic levels of H_2O_2, which, despite not being a highly reactive molecule, can give rise to oxidative stress (Pizzinat, et al., 1999; Tipton, 2018). CHE is an essential enzyme in the body's redox balance because if it is high, it can reduce melanin synthesis and favor the onset of vitiligo (Schallreuter, et al., 2004; Schallreuter & Elwary, 2007). CWT, butin, and galangin can promote

a decrease in the content of MDA and levels of CHE in the blood and skin of animals. Abuduaini, et al., 2021, also showed a decrease in MAO by CWT. These results suggest that redox balance restoration stands out among the possible mechanisms by which CWT, butin, and galangin act in treating vitiligo. Another compound capable of restoring the redox balance recorded in articles included in this review was RY3-c. The tests performed showed that it was able to decrease the ROS content in a dose-dependent manner.

Melanogenesis is mediated by several signaling pathways, including the p38 mitogen-activated protein kinase (MAPK) signaling pathway (Kim, et al., 2015), Akt/GSK-3β/β-catenin pathway (Zang, et al., 2019), NRF2 signaling pathway, α-MSH/cAMP and the PI3K/Akt signaling pathway (Hwang, et al., 2019; Pillaiyar, et al., 2017). The NRF2 signaling pathway is fundamental in protecting melanocytes against damage triggered by H_2O_2 because NRF2 maintains the cell's correct redox balance. It works as a transcription factor to regulate the expression of several antioxidant defense genes (Itoh, et al., 1999; Niture, et al., 2014).

Under normal conditions, NRF2 is located in the cytosol in a complex with Keap-1, but at the signal of oxidative stress, NRF2 is released and moves to the cell nucleus. In the nucleus, it combines with antioxidant response elements, and antioxidant enzymes are activated (Guo & Zhang, 2021). Among the reactive nitrogen species, nitric oxide is the main substance in tissue, and inducible nitric oxide synthase is largely responsible for stimulating cells to produce NO (Mansourpour, et al., 2019; Xia, et al., 2019). Thus, if a compound can perform the upregulation of NRF2 and downregulation of nitric oxide synthetase, it contributes to the restoration of redox balance, as CAP (Zhai, et al., 2021).

The MAP family is composed of p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), which, after phosphorylation, can activate MITF, and thus the cascade common to all pathways for melanin synthesis is triggered (Wu, et al., 2011). MAPK can be indirectly activated by the alpha-MSH hormone, which binds to the melanocortin 1 receptor (MC1R), and by stem cell factor (SCF), which binds to the c-kit receptor on the surface of the melanocyte (Gonzalez & Montminy, 1989). RY3-c acted mainly in the p38-MAPK and ERK1/2 pathways and played an important role in melanin synthesis (Pillaiyar, et al., 2017).

Histopathological assays performed by Abuduaini, et al., 2021, Huo, et al., 2017, and Huo, et al., 2014 showed that CWT, butin, and galangin induced an increase in the number of basal melanocytes and epidermal cells. In addition, galangin, CWT, butin, 6-bap, and the ratio butin:caffeic acid:luteolin (1:4:10) promoted upregulation of TYR expression. Other upregulated genes found in this review were TRP-1, by CWT, butin, galangin, and 6-bap; TRP-2 by CWT; and mitfa, another prominent gene in the cascade, regulated by 6-bap and butin:caffeic acid:luteolin (1:4:10).

The MITF and TYR genes are already well defined as melanogenic genes (Niu & Aisa, 2017). In addition to them, during the early color development of zebrafish, the dct and kit genes promote the differentiation of melanocyte stem cells into melanocytes (Rawls & Johnson, 2000). Starting from this point, the effects of the best combination of butin: caffeic acid: luteolin (1:4:10) on the expression of these melanogenic genes were investigated. The investigation concluded that the expression of tyr, kit, and dct in the eyes and on the back of zebrafish larvae increased, whereas the expression of MITF was only slightly increased on the back of the fish (Lai, et al., 2021). Thus, the increase in gene expression of tyr, kit, and dct is responsible for the increase in melanin production in zebrafish larvae treated with the combination of these compounds.

5. Conclusion

The studies show that the mechanisms of the antioxidants evaluated in these works, within *in vivo* models, included, mainly, the increase of tyrosinase activity by increase in the synthesis of TYR and TRP-1 proteins, the positive regulation of the number of epidermal cells, the restoration of redox balance, MITF activation, decrease in malondialdehyde content and

decrease in cholinesterase levels and activity. The results suggest that compounds that have not yet been clinically tested should advance in this direction, as the *in vivo* results are promising. Furthermore, it was observed the total or partial absence of some information about the methodology used on the reported *in vivo* studies, evidenced by the evaluation of the risk of bias, showing the importance of a criterious description of the methodology of the studies to strength the confidence in the results reported.

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