ORIGINAL ARTICLE

Enzymatic Transglycosylation Reaction of Carotenoid Glycoside and Its Functional Properties as Antioxidant and Antimutagen

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ABSTRACT

Introduction: Naturally occurring carotenoids often exhibit limited solubility and stability, which restrict their functional applications despite their strong antioxidant and health-promoting potential. Enzymatic glycosylation has been proposed as a strategy to enhance the bioactivity of carotenoids. This study aimed to evaluate *Monascus purpureus* as a novel source of crude enzymes with transglycosylation activity for the synthesis of carotenoid glycosides (CGs), and to compare their functional properties with the native carotenoid aglycones. **Materials and Methods**: Starch was used as a donor substrate, and carotenoids from *M. purpureus* cell cultures served as acceptors in the transglycosylation reaction. The resulting carotenoid glycosides were purified by ODS gel column chromatography and characterized using TLC and HPLC. **Results:** Antioxidant activity was evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, yielding IC_{50} values of 109.59 ± 4.15 µg/mL for carotenoid glycoside and 137.18 ± 5.05 µg/mL for the carotenoid aglycone. These results indicate that glycosylation improved antioxidant activity by approximately 25.17%. Antimutagenic activity was also enhanced, with the carotenoid glycoside showing 97.1% inhibition of mutagens, compared with 71.6% for the aglycone. **Conclusion:** Compared with commercial astaxanthin and β-carotene, both microbial carotenoids and their glycosylated derivatives exhibited higher antimutagenic activity. These findings suggest that enzymatic transglycosylation using *M. purpureus* enzymes can be an effective approach to enhance the functional value of carotenoids for potential nutraceutical applications.
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Keywords: Transglycosylation, Carotenoid glycoside, Antioxidant, Antimutagen, *Monascus purpureus*

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INTRODUCTION

Enzymatic transglycosylation offers several advantages over chemical glycosylation—such as fewer steps, reduced steric hindrance, regiospecificity, cost-effectiveness, mild reaction conditions, and environmental friendliness—while chemical approaches often require complex protective group strategies [1]. This enzymatic approach also enhances solubility and oxidative stability of bioactive compounds [2]. Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19), an α-amylase family enzyme, catalyzes multiple reactions—including cyclization, coupling, and disproportionation—to yield glycosylated carotenoids that are more light- and oxidation-resistant than their aglycone forms [3–6].

Monascus purpureus, a filamentous fungus traditionally used in producing red yeast rice, generates a rich

array of secondary metabolites such as monacolin K, enzymes, and notably, carotenoids—many of which exhibit potent antioxidant, anticancer, antimutagenic, anti-inflammatory, and cholesterol-lowering effects [7]. Among its pigments, compounds like monascin and ankaflavin show strong antioxidant activity, while monasfluore B exhibits antimutagenic potential; IC_{50} values for certain pigments range from ~62 to 80 µg/mL [8,9]. Additionally, fermentation with *M. purpureus* has been shown to enhance total polyphenols, flavonoids, and antioxidant capacity—as demonstrated in ginger substrates analyzed via DPPH, ABTS, and FRAP assays [10].

This study aimed to synthesize carotenoid glycosides (CGs) via enzymatic transglycosylation catalyzed by CGTase derived from *M. purpureus*, using carotenoid extracts as acceptor substrates. The antioxidant and antimutagenic activities of the glycosylated products were assessed to evaluate their functional enhancement compared with carotenoid aglycones.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Monascus purpureus was obtained from laboratory stock culture and maintained on potato dextrose agar (PDA) slants at 4°C. For carotenoid production, the strain was cultivated in liquid fermentation medium containing glucose (40 g/L), peptone (10 g/L), KH_2PO_4 (2 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), and yeast extract (5 g/L), adjusted to pH 6.0 prior to sterilization. Cultures were incubated at 30°C under static conditions for 7–10 days until sufficient pigmentation developed, following methods previously described for enhancing *Monascus*-derived carotenoid biosynthesis [11,12]. For testing mutagenic activity, the mutant strain *Salmonella typhimurium* TA98 was acquired from JIRCAS, Japan.

Preparation of Crude Enzyme Extract

The *M. purpureus* culture was cultivated in a medium consisted of 30 g of glucose, 0.5 g of yeast extract, 1.0 g of tryptone, 1.8 g of NH4Cl, 2 g of KH2PO4, 0.5 g of MgSO4·7H2O, 0.035 g of FeSO4·7H2O, 0.007 g of MnSO4·7H2O, 0.011 g of ZnSO4·7H2O, 0.005 g of CuSO4·7H2O, 0.002 g of CoCl2·5H2O, 0.0013 g of Na2MoO4·2H2O, 0.002 g of H3BO4, and 0.0005 g of Al2(SO4)3. To stimulate enzyme production, 20 g of starch in a buffer at pH 6.0 was added, and the mixture was sterilized by autoclaving at 121°C for 15 min. The medium was then inoculated with the fungal strain and incubated on a rotary shaker at >120 rpm and 37°C for several days [3]. Crude enzymes with potential transglycosylation activity were extracted by suspending the biomass in 50 mM sodium phosphate buffer (pH 6.0), followed by homogenization and centrifugation at 10,000 × g for 20 min at 4°C. The supernatant containing crude enzymes was collected and used directly for transglycosylation reactions.

Extraction of Carotenoid Compound

Carotenoid compounds were extracted from the culture of *M. purpureus* based on a modified method [13,14] to obtain its cell pellets. Next, the cell pellet was added to 75% ethanol and sonicated for 90 min, then centrifuged at 3000xg for 15 min [14]. Furthermore, the supernatant containing Carotenoid compounds in ethanol was separated from the precipitate in the form of cell pellet residue, then concentrated using a rotary evaporator and then freeze-dried using a freeze dryer to obtain carotenoid powder, which was stored at -20°C to be used as the acceptor in the synthesis of CG by application of enzymatic transglycosylation reaction [15].

Measurement of Cell Biomass

Carotenoid compounds were extracted from *M. purpureus* cultures using a modified procedure [13,14] to obtain cell pellets. The cell pellets were suspended in 75% ethanol, sonicated for 90 min, and then centrifuged at 8000 rpm for 15 min [15]. The supernatant, which contained the carotenoid compounds in ethanol, was separated from the cell pellet residue. It was then concentrated using evaporator to produce a microbial carotenoid powder, which was used as the acceptor in the synthesis of CG through transglycosylation reaction.

The growth of fungal mycelia was evaluated by determining the dry weight of mycelia, following the method described in reference [16]. To obtain mycelial pellets, 5.0 mL of culture medium was transferred to a 15 mL tube, washed twice with distilled water, and centrifuged at 10,000 rpm for 10 min. The collected mycelia were then dried at 80°C for 36 hours and then weighed. The specific growth rate of *M. purpureus* was estimated using the formula:

$$\mu = \frac{\ln(N_1) - \ln(N_0)}{T_1 - T_0}$$

where N_0 and N_1 represent the initial and final biomass (g), respectively, and T0 and T1 denote the initial and final times (h).

Analysis of Total Carotenoids

The total fungal carotenoids extracted from the fungal strain were analyzed using high-performance liquid chromatography on a Shimadzu LC-20AB system. The analysis was performed with a Spherisorb ODS-C18 column (250 × 4.6 mm, 5 µm particle size, 100 E pore size). The mobile phase was an ethyl acetate gradient (0–100%) in a hexane-acetone solution (85:15, v/v), flowrate at 2 mL/min and maintained at 20°C. A photodiode array detector recorded the absorbance spectrum for each peak. Carotenoid peaks were identified based on their retention times at 470 nm. A standard solution of Carotenoids, purchased from Sigma was used to identify absorbance peaks of these compounds [14,15].

Substrate Preparation and Transglycosylation Reaction
The reaction mixture consisted of soluble starch (10 mg/mL) as the glycosyl donor and *M. purpureus* carotenoid extract (5 mg/mL in ethanol) as the acceptor. The mixture was incubated with crude enzyme at 40 °C for 24 h under gentle agitation. The reaction was terminated by boiling for 5 min, followed by centrifugation at 8,000 × g for 10 min. The supernatant containing the carotenoid glycoside (CG) was collected. This enzymatic approach was adapted from established studies on microbial carotenoid transformation [11,13].

Purification of Carotenoid Glycosides
The CG fraction was purified by octadecylsilane (ODS) gel column chromatography using a gradient of methanol:water (70:30 → 100:0, v/v). Fractions exhibiting strong absorption at 470 nm (UV–Vis spectrophotometer) were pooled and concentrated under reduced pressure. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were employed for characterization and confirmation of glycosylated carotenoids, as reported by Yang et al [12]. The transfer product obtained from the reaction mixture was analyzed using thin-layer chromatography with a mobile phase of ethyl acetate-acetic acid-distilled water (3:1:1, v/v). The TLC plate was visualized by spraying with 50% H_2SO_4 in methanol, and then heated at 100°C for 15-20 min to enhance visualization [17].

Assay for Antioxidant Activity
The antioxidant activity of the CG produced through transglycosylation was assessed following the method described [18], using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. A 0.1 mM DPPH solution was prepared by dissolving approximately 4.0 mg of DPPH in methanol. Serial dilutions of carotenoid glycoside and carotenoid aglycone were prepared, and absorbance was measured at 517 nm after 30 min incubation in the dark using a UV-Vis spectrophotometer (Lambda 25, Perkin Elmer) and compared with controls containing ascorbic acid. The half-maximal inhibitory concentration (IC_{50}) was calculated using a nonlinear regression curve, expressed as µg/mL [11]. A decrease in absorbance indicates enhanced free radical scavenging activity against the oxidizing agents [19,20].

Preparation of Aflatoxin Extract and S-9-Mix
In a test tube, 10 mL of GY medium was prepared, containing 25% glucose, 5-7% yeast extract, and distilled water, and sterilized by autoclaving at 121°C for 15 min. A culture of Aspergillus sp. was then inoculated into the GY medium and incubated at 25-30°C for a week in a dark room. After incubation, the medium containing the fungal culture was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected and extracted using chloroform (1:1, v/v). The chloroform extract was transferred to a conical flask and shaken for several min. The precipitate as chloroform layer was then isolated and placed into a glass bottle. The chloroform residue containing the aflatoxin extract was then evaporated in a water bath [22].

To prepare the S-9 mix solution, 50 g of mashed chicken liver was blended with 150 mL of distilled water until a homogeneous mixture was achieved. This mixture was sterilized at 121°C for 15 min, then centrifuged at 9000xg for 10 min at 4°C. The resulting supernatant, containing the S-9 mix solution, was separated and stored at a low temperature for later use [22].

Assay for Antimutagenic Activity
The antimutagenic activity of CG against Trp-P-1 in *S. typhimurium* TA98 was evaluated using pre-incubated aflatoxin extract in the S9-mix [21]. The antimutagenicity assay followed the procedure outlined [22]. To start, 2.0 mL of top agar medium at 45-50°C was prepared in a medium containing 0.5% NaCl, 0.65% agar, and 1.0 mL of a 0.5 mM Biotin-Histidine solution. This medium also included 0.05 mL of aflatoxin extract as mutagen and CG extract as antimutagen. Then, approximately 0.05 mL of *S. typhimurium* TA98 culture was added to the top agar and poured into a petri dish. Furthermore, an E-minimum agar medium containing 1.65% agar and 2.0% glucose in a Vogel-Bonner salt solution (consist of Mg.SO4·7H2O, citric acid, anhydrous KH_2PO_4, and Na2NH2PO4·4H2O). This E-minimum agar was poured over the top agar layer in a petri dish. The setup was then incubated at 35-40°C for 2 days. After incubation, the number of colonies on the medium was counted to determine the antimutagenic activity of CG against Trp-P-1 in *S. typhimurium* TA98.

RESULTS

Qualitative and Quantitative Extraction and Determination of Carotenoid
The cultivation of *M. purpureus*, can produce pigments in yellow, orange, and red hues, influenced by the culture conditions and the carbon-to-nitrogen ratio in the medium [23] as shown in Fig. 1. Supplementing the medium with nitrogen compounds enhanced some water-soluble pigment production [24]. The reddish carotenoid extract served as the donor for a glycosyltransferase enzyme-ctalyzed transglycosylation reaction, yielding CG [1].

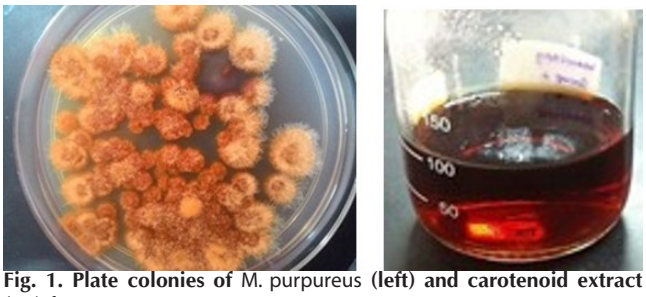

Fig. 1. Plate colonies of M. purpureus **(left) and carotenoid extract (CE) from** M. purpureus

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Enzymatic transglycosylation Reaction Carotenoid Glycoside

The enzymatic reaction with CGTase extracted from *M. purpureus* synthesized carotenoid glycoside (CG) via the transfer of glucose residues to acceptors with hydroxyl (OH) groups. Thin-layer chromatography (TLC) analysis confirmed the presence of transglycosylation products (CG), hydrolysis products (G, M), the reference glycoside (Ar, arbutin), and residual carotenoid extract (CE) (Fig. 2A). The CG were detected as fluorescent spots under UV light at 365 nm and visualized after sulfuric acid treatment.

The RF values of the newly formed glycosylated products were different from those of the donor carotenoid extract but close to the RF value of the standard glycoside (arbutin), thereby confirming the successful synthesis of carotenoid glycoside (CG) (Fig. 2B). TLC analysis demonstrated that the enzyme preparation contained both hydrolytic and transglycosylation activities. Glucose (G) and maltose (M) indicated hydrolysis products, while distinct CG spots corresponded to transglycosylation products [12].

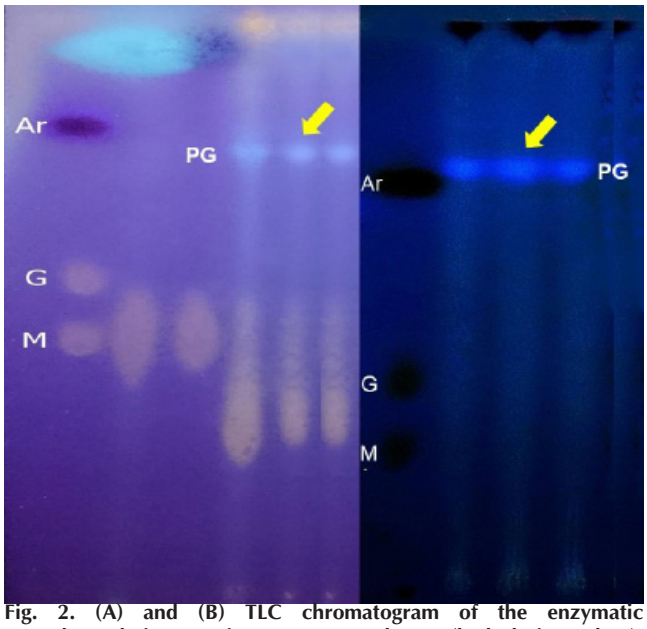

Fig. 2. (A) and (B) TLC chromatogram of the enzymatic transglycosylation reaction. Lanes: G = glucose (hydrolysis product); M = maltose (hydrolysis product); Ar = arbutin (commercial glycoside standard); CE = carotenoid extract; CG = carotenoid glycoside (transglycosylation product). (A) crude reaction mixture before purification; (B) purified reaction mixture after diethyl ether extraction.

Radical Scavenging Activity Against DPPH

The radical scavenging ability of CG against DPPH was assessed and compared with that of carotenoid extract (CE) and ascorbic acid. As shown in Fig. 3 and summarized in Table I, the IC_{50} value of CG was 109.59 µg/mL, compared to 137.18 µg/mL for CE and 38.98 µg/mL for ascorbic acid. Contrary to the earlier description, CG did not exhibit stronger antioxidant activity than ascorbic acid. Instead, ascorbic acid showed the highest potency, while CG displayed stronger radical scavenging activity than CE. These findings suggest that glycosylation enhanced the antioxidant potential of carotenoids relative to their crude extract but remained less effective than the standard ascorbic acid.

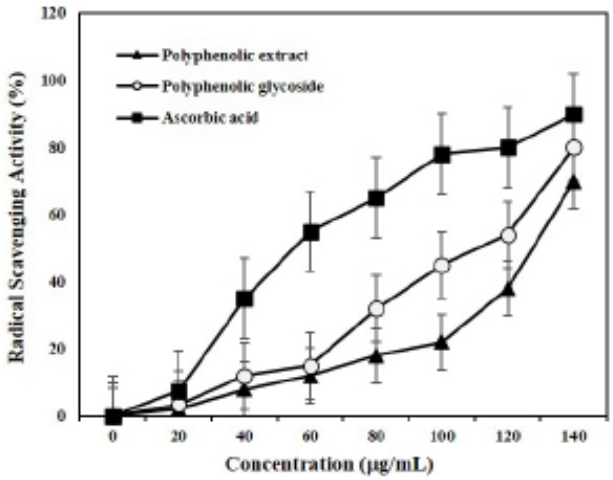

Fig. 3. Radical scavenging capacity (DPPH assay) of carotenoid glycoside (CG), carotenoid extract (CE), and ascorbic acid (AA). IC_{50} values (µg/mL) calculated from the inhibition curves and error bars indicate standard deviations.

Table I. IC_{50} values of CG, CE, and AA against DPPH radicals.

Samples	IC_{50} Value (µg/mL)
Carotenoid glycoside	109.59 ± 4.15[b]
Carotenoid aglycone	137.18 ± 5.05[a]
Ascorbic acid (control)	38.98 ± 6.43[c]

Antimutagenic Properties of Carotenoid Glycoside

The antimutagenic activity of CG was evaluated in *S. typhimurium* TA98 exposed to aflatoxin. As summarized in Table II, neither CE nor CG induced mutant colonies in the absence of aflatoxin. In contrast, aflatoxin alone markedly increased revertant colonies (positive control: 124.33±7.77 colonies), confirming its mutagenic potential. Both CE and CG significantly inhibited the mutagenic effect of aflatoxin. CE inhibited 71.6% of aflatoxin-induced mutations, while CG exhibited 97.1% inhibition, likely due to the enhanced biological and pharmacological properties associated with glycosylated carotenoids over their aglycone forms [26]. The negative control (no mutagen) showed 2.67±1.15 revertants. These results indicate that glycosylation enhanced the antimutagenic activity of carotenoids.

Table II: Antimutagenic properties of carotenoid extract (CE) and carotenoid glycoside (CG) compared to arbutin against aflatoxin-induced mutations in S. typhimurium **TA98.**

Samples	Revertant colony (CFU)	Inhibition (%)
Negative control	124.33 ± 7.77[c]	ND
Positive control	2.67 ± 1.15[a]	ND
Polyphenolic extract	35.33 ± 9.07[b]	71.6
Polyphenolic glycoside	3.67 ± 1.15[a]	97.1
Commercial glycoside	1.33 ± 0.58[a]	98.9

*ND: not determined.

As shown in Fig. 4, commercial glycosides (arbutin), as well as other carotenoids (β-carotene, astaxanthin), demonstrated antimutagenic activities of 58.21%, 35.82%, and 59.70%, respectively. The synthesized CG demonstrated superior inhibition (86.57%) compared to these commercial compounds. This suggests that CG exhibited stronger antimutagenic effects than the crude CE. Importantly, the term carotenoid extract (CE) is used here to represent the crude extract, which has not been further characterized for specific aglycone composition.

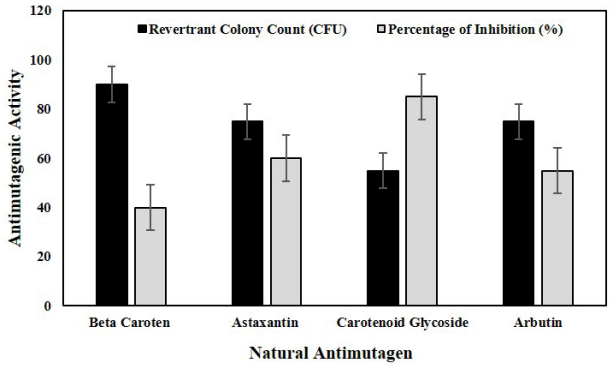

Fig. 4. Antimutagenic efficacy of synthesized carotenoid glycoside (CG) compared with commercial compounds. Bars represent mean inhibition percentages; error bars = SD. β-carotene, astaxanthin, CE, CG, and arbutin were tested against sodium azide in S. typhimurium **TA98.**

DISCUSSION

Carotenoids are prone to oxidative degradation, light exposure, and enzymatic processes, which reduce their stability and bioactivity. Glycosylation improves carotenoid stability by enhancing solubility and resistance to oxidation, thereby preserving their biological functions [27]. Enzymatic glycosylation is advantageous over chemical synthesis, which requires multiple protection–deprotection steps and tight control of anomeric configuration [28]. The results confirm that *M. purpureus* can produce carotenoid glycosides (CG) via CGTase-mediated transglycosylation, representing an efficient and selective alternative for generating stable bioactive carotenoid derivatives.

TLC analysis confirmed the presence of distinct transglycosylation products compared to the carotenoid extract. The RF values of the synthesized CG matched that of the reference glycoside arbutin, consistent with previous reports where TLC was used to verify glycosylated products of phenolics and carotenoids [25]. The visualization of fluorescent spots under UV light further supported successful glycosylation. Similar approaches were reported by Yang et al. [12], where TLC was employed to distinguish glycosides from hydrolysis by-products, validating the reliability of this method for rapid identification of glycosylated compounds.

Unlike chemical synthesis, which often requires multiple protection–deprotection steps to control anomeric configuration and regioselectivity, enzymatic glycosylation offers high stereo- and regiospecificity without protective groups. Through transglycosylation, insoluble and unstable bioactive compounds can be converted into more soluble and stable derivatives in a simple biological process [2]. For instance, glucosides of bioactive molecules such as vitamin glucosides have been reported as effective anti-allergic agents [29].

In nature, glycosidic bonds are formed by glycosyltransferases, which transfer activated sugar donors to acceptor molecules. Despite their potential in biocatalysis, large-scale use of glycosyltransferases is limited by the high cost of sugar-nucleotide donors and challenges in expressing these membrane-bound enzymes [30]. Alternatively, glycosidases are widely available across organisms and can be exploited for synthesis through reverse hydrolysis or transglycosylation, in addition to their natural hydrolytic activity [31–33].

The antioxidant capacity of a compound is commonly expressed as its IC_{50} value, which represents the concentration required to inhibit 50% of radical activity. A lower IC_{50} corresponds to stronger scavenging potential. In the DPPH assay, this radical remains stable due to electron delocalization, giving it a deep purple color with absorption at 520 nm. Upon reaction with hydrogen donors, DPPH is reduced, leading to a measurable decrease in absorbance proportional to antioxidant concentration [34,35].

In terms of antioxidant activity, the CG exhibited a significantly lower IC_{50} than the carotenoid extract, indicating improved radical scavenging capacity. Although their IC50 did not surpass that of ascorbic acid, CG clearly enhanced antioxidant activity relative to the aglycone extract. Comparable findings have been reported for glycosylated carotenoids from microbial and plant sources, where glycosylation improved stability and antioxidant potential compared to non-glycosylated forms [36,37]. These results suggest that enzymatic glycosylation may extend the functional application of carotenoids in mitigating oxidative stress.

The antioxidant activity of carotenoid glycoside and its aglycone was evaluated using the DPPH radical

scavenging assay. The glycosylated carotenoid exhibited significantly higher scavenging activity compared to the non-glycosylated form, with an IC_{50} value of 109.59 ± 4.15 µg/mL, representing a 25.17% improvement in antioxidant potential. This enhancement can be attributed to the increased solubility and stability conferred by glycosylation, which facilitates better interaction with free radicals. Similar improvements in radical scavenging activity upon glycosylation have also been reported for flavonoids and carotenoids, where glycoside derivatives showed higher antioxidant efficiency due to structural modifications that enhanced their electron-donating ability [38,39].

The findings from this study are consistent with previous reports, such as De Winter et al. [38] and Liang et al. [39], who demonstrated that enzymatic glycosylation of carotenoid derivatives significantly improved their radical scavenging properties compared to the parent aglycones. These results collectively support the notion that glycosylation not only enhances the physicochemical properties of bioactive compounds but also their biological functionality, particularly in terms of free radical neutralization.

The glycosylated carotenoid exhibited higher radical scavenging activity compared to its aglycone form, consistent with previous studies reporting that glycosylation can enhance the stability and antioxidant properties of carotenoids and polyphenols [38].

The CG also demonstrated superior antimutagenic activity compared to the carotenoid extract. Inhibition of aflatoxin-induced mutations in *S. typhimurium* TA98 reached 97.1% with CG, compared to 71.6% for the extract. This level of inhibition was comparable to arbutin and greater than commercial antioxidants such as β-carotene and astaxanthin. Similar antimutagenic effects of carotenoid derivatives have been observed in *Chlorococcum humicola* extracts, which reduced mutagenicity of aflatoxin and other carcinogens in Ames assays [40,41]. These findings confirm that glycosylation enhances the biological potential of carotenoids, supporting their role as natural antimutagens with pharmaceutical relevance.

Overall, this study highlights the dual antioxidant and antimutagenic potential of enzymatically synthesized CG from M. purpureus. The improved stability and bioactivity compared with the aglycone extract and some commercial compounds emphasize the advantages of enzymatic transglycosylation in producing functional carotenoid derivatives. These properties suggest applications of CG in functional foods, nutraceuticals, and natural chemopreventive agents.

CONCLUSION

This study successfully synthesized carotenoid glycoside through enzymatic transglycosylation using cyclodextrin glucanotransferase (CGTase) derived from *Monascus purpureus*, resulting in enhanced bioactivity. The glycosylated carotenoid exhibited a 25.17% increase in antioxidant activity (IC_{50}: 109.59 ± 4.15 µg/mL) and significantly improved antimutagenic effects, achieving 97.1% inhibition of mutagens compared to 71.6% for the non-glycosylated carotenoid (aglycone). These results demonstrate that glycosylation improves the radical scavenging capacity and protective potential of carotenoids, positioning carotenoid glycoside as a promising candidate for cancer prevention and functional food applications. Furthermore, its potential as a natural alternative to synthetic additives underscores its relevance in pharmaceuticals and health supplements. Future studies should focus on elucidating the underlying mechanisms of action and evaluating efficacy in various biological systems to further establish the role of carotenoid glycosides in mitigating genetic damage and promoting human health.

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