RADICAL-SCAVENGING ACTIVITY OF $(KLAKLAK)_2$ BIOCONJUGATES WITH CAFFEIC ACID

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ABSTRACT

Free oxygen radicals released in the body because of various metabolic processes could lead to the so-called oxidative stress in cells, which is a precursor to various types of cancer. Protecting cells from oxidative stress is an important step in the prevention of cancer. Nature has a large supply of compounds with powerful antioxidant properties. One of them is caffeic acid. In the present study, caffeic acid was combined to obtain bioconjugates with analogues of the natural peptide with proven antitumor properties $(KLAKLAK)_2$.

Here we report the antioxidant potential of these molecules as it was investigated with the well-known from the literature DPPH method, in search of a synergistic effect between the two pharmacophores of the molecule. The obtained results identify Si_{18} $(Caf\text{-}(KnLAKnLAK)_2\text{-}NH_2)$ as the lead candidate approaching the caffeic acid benchmark under the tested conditions with EC_{50} = 0.0178 mM.

Keywords: antitumor peptides, antioxidant peptides, caffeic acid, DPPH method.

INTRODUCTION

The free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) was firstly synthesized in 1922 by Goldschmidt and Renn [1]. Further, Blois established its application as a model radical for evaluating antioxidant activity [2]. Methodological refinements by Brand-Williams et al. subsequently standardized the assay and contributed to the widespread adoption as a reference method for radical-scavenging evaluation [3].

DPPH• is a stable nitrogen-centered radical whose persistence is attributed to extensive delocalization of the unpaired electron over the aromatic system, further stabilized by the electron-donating diphenylamino group and the electron-withdrawing picryl substituent (Fig. 1) [4, 5].

This configuration prevents dimerization and accounts for the intense violet coloration of the radical, with a characteristic absorption maximum near 517 nm in alcoholic solutions [6, 7].

Upon reaction with antioxidants capable of donating a hydrogen atom or an electron, DPPH• is reduced to the corresponding hydrazine (DPPH-H), accompanied by a visible colour change from violet to pale yellow. This colorimetric change provides the basis for the DPPH assay, which is readily monitored by UV-VIS spectrophotometry [6, 8 - 14].

Due to its simplicity, rapid execution, and high reproducibility, the DPPH assay is among the most widely applied methods for evaluating radical-scavenging activity. It has been extensively employed

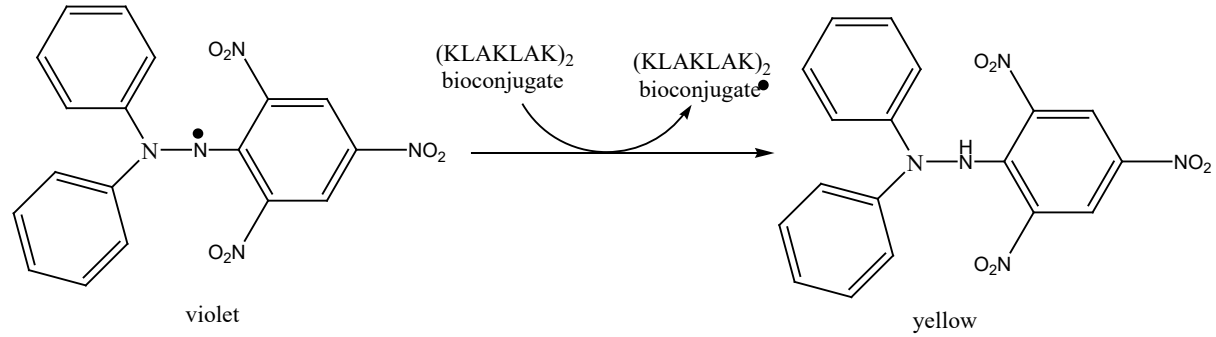

Fig. 1. Reaction of radical scavenging in the presence of $(KLAKLAK)_2$ analogues.

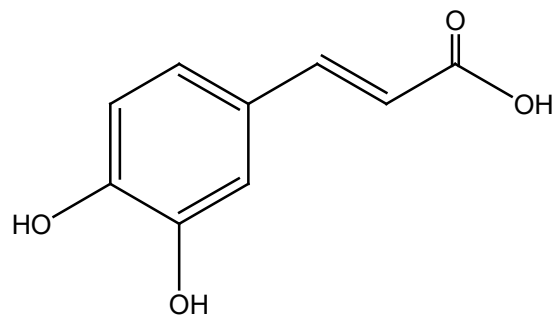

Fig. 2. Caffeic acid structure.

for the assessment of bioactive molecules, providing a reliable tool for both qualitative and quantitative characterization of antioxidant potential [6, 7, 15].

(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid, also known as hydroxycinnamic acid or caffeic acid (Fig. 2) is well known compound with antioxidant, anti-inflammatory, anticancer and many other activities [16, 17].

Among phenolic antioxidants, caffeic acid is widely used as a reference standard in DPPH assays, with the radical-scavenging activity consistently demonstrated in standardized protocols and primary studies [11, 15, 18 - 20].

Bioactive peptides have emerged as an important class of radical scavengers, with numerous studies demonstrating their *in vitro* antioxidant activity as quantified by the DPPH assay [21 - 27].

The present study aims to evaluate for the first time the radical-scavenging capacity of $(KLAKLAK)_2$-bioconjugates using the DPPH assay. To allow comparative interpretation, caffeic acid was included as a benchmark phenolic antioxidant.

EXPERIMENTAL

Materials

Absolute ethanol (99 % v/v) was obtained from Valerus Ltd. (Sofia, Bulgaria). 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and caffeic acid (≥ 98%) were purchased from Sigma-Aldrich (Darmstadt, Germany). All reagents were of analytical grade and used as received.

The peptides used in this study were Si_1, Si_8, Si_{10}, Si_{11}, Si_{12}, Si_{15}, and Si_{18}, with general structures summarized in Table 1. Synthesis of targeted peptides was previously described by Jaber et al. [28 - 30].

Table 1. Peptides evaluated and their nominal structure.

Code	Peptide structures	Code	Peptide structures
Si_1 (parent peptide)	$(KLAKLAK)_2$-NH_2	Si_{12}	Caf-(KLβ-AKLβ-AK)$_2$-NH_2
Si_8	Caf-KLAKLAK-NH_2	Si_{15}	Caf-KnLAKnLAK-NH_2
Si_{10}	Caf-$(KLAKLAK)_2$-NH_2	Si_{18}	Caf-$(KnLAKnLAK)_2$-NH_2
Si_{11}	Caf-KLβAKLβAK-NH_2		

Methods

DPPH method

Antioxidant activity of the peptide set was quantified by the DPPH radical-scavenging assay in absolute ethanol. Peptide stock solutions (20 mM) were prepared in advance and diluted with absolute ethanol to the working concentrations listed in Table 2.

A 0.10 mM DPPH solution in absolute ethanol (99.9 % v/v) was prepared and adjusted to an absorbance of 0.80 - 0.90 at 517 nm. Measurements were performed in disposable polystyrene cuvettes (3 mL capacity, 10 mm optical path length) using a T70 UV/Vis spectrophotometer (PG Instruments Ltd). For each determination, 1.50 mL of the DPPH working solution was mixed with 0.05 mL of peptide solution. The control contained 1.50 mL DPPH solution and 0.05 mL ethanol, and the solvent blank consisted of ethanol alone. Absorbance was recorded at 0, 15, 30, 45, and 60 min. Radical-scavenging response was expressed using the following equations 1 and 2 [13]:

$$Quenched\ DPPH\bullet = \frac{A_c - A_s}{A_c}\ 100,\% \quad (1)$$

$$Remnant\ DPPH\bullet = 100 - \%\ quenched\ DPPH\bullet,\% \quad (2)$$

where A_C and A_S denote to the control and sample absorbances at the time point of 60 minutes. Antioxidant activity was further characterized by EC_{50}, defined as the concentration required to quench 50 % of the initial DPPH• signal [31 - 35]. Each condition was measured in five replicates. Results are reported as mean ± SD (standard deviation). For % quenched and % remnant, the SD did not exceed 3.6 percentage points.

RESULTS AND DISCUSSION

As shown in Fig. 3, all analytes reacted rapidly with DPPH• and reached an apparent plateau by 30 min, except for the parent peptide Si_1. The observation of a 30 min stabilization is consistent with commonly used protocols that specify incubation for 30 min at 517 nm in ethanolic media [36 - 38]. For quantitative comparisons a fixed 60 min endpoint was adopted, aligning with methodological work that selected 60 minutes for subsequent tests after kinetic appraisal and noted 30 - 60 min as common practice [39].

For Si_1, even at the highest concentration tested

Table 2. Working concentrations, mM) of the peptides and caffeic acid used in the DPPH radical-scavenging assay.

Analyte	Working concentration, mM
Si_1 (parent peptide)	20, 10
Si_8	2, 1, 0.4, 0.2
Si_{10}	2, 1, 0.8, 0.4, 0.2
Si_{11}	20, 10, 5
Si_{12}	10, 2, 1, 0.8, 0.2
Si_{15}	1, 0.8, 0.6
Si_{18}	1, 0.5, 0.1
Caffeic acid	0.61, 0.46, 0.31, 0.18

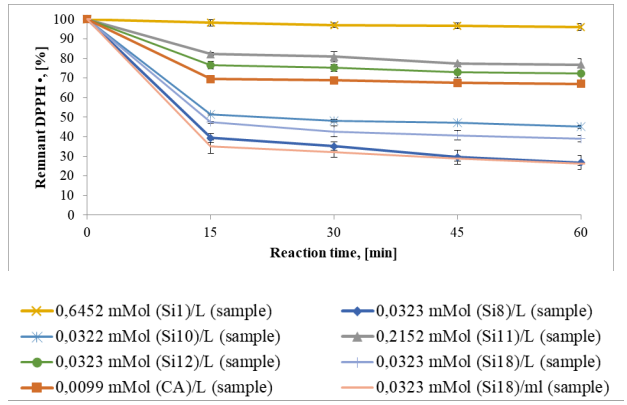

Fig. 3. DPPH• scavenging kinetics for the peptide series and caffeic acid.

(0.6452 mM), only a 4 % decrease in remnant DPPH• was observed over the 60 min interval, which is consistent with the absence of a redox-active phenolic unit (such as the catechol group of caffeic acid) capable of efficient hydrogen-atom or electron donation and resonance stabilization of the resulting radical [40].

The concentration-response data for DPPH• quenching was fitted to a second-order polynomial (Eq. (3)):

$$y = aC^2 + bC \quad (3)$$

where y denotes % quenched DPPH• and C the final in-cuvette analyte concentration (mM). The fitted parameters a and b and the corresponding coefficients of determination (R^2) are summarized in Table 3, with $R^2 \geq 0.9914$ in all cases.

EC_{50} values at the 60-minute endpoint were obtained by solving y(C) = 50 for *C*. EC_{50} served as the primary metric for comparing radical-scavenging capacity across the peptide series.

Table 3. Radical-scavenging capacity of the peptides and caffeic acid, expressed as EC_{50}, mM in the DPPH assay.

Sample name	a	b	Ec_{50}, mM	R^2
Si_1	-20.99	19.581	2.079 *	1.000
Si_8	-29.931	3117.4	0.0191	0.9999
Si_{10}	-10911	2033	0.0292	0.9982
Si_{11}	-29.566	118.55	0.479	0.9993
Si_{12}	-1720.2	836.02	0.0698	1.000
Si_{15}	37860	709.76	0.0282	0.9914
Si_{18}	-35756	3440.2	0.0178	0.9999
Caffeic acid	-14781	3527.8	0.0151	0.9981

**Extrapolated value*

For Si_1, the EC_{50} estimate required extrapolation beyond the tested concentration range. Across the peptide series, Si_{18} exhibited the greatest activity at the 60 min endpoint (EC_{50} = 0.0178 mM). It was about 117 times stronger than the parent peptide Si_1 (EC_{50} = 2.079 mM, extrapolated) and about 1.18 times weaker than the positive control, caffeic acid (EC_{50} = 0.01513 mM). Si_{11} was the least active peptide at the 60-min endpoint (EC_{50} = 0.479 mM). Against the positive control, caffeic acid (EC_{50} = 0.01513 mM), this corresponds to about 32 times weaker activity, whereas relative to the parent peptide Si_1 (EC_{50} = 2.079 mM) it remained about 4.3 times stronger.

CONCLUSIONS

This study successfully characterized the antioxidant strength of KLAKLA-derived peptides using the DPPH assay. Reaction profiles reached an apparent plateau by 30 min and a fixed 60 min endpoint was adopted for all quantitative comparisons. Percent-quenched DPPH• responses were fitted to a second order polynomial, yielding coefficients of determination $R^2 \geq 0.9914$, and replicate standard deviations did not exceed 3.6 %.

A clear rank order across the series was established by the EC_{50} values. The greatest activity was observed for Si_{18}, with EC_{50} = 0.0178 mM. This corresponded to approximately 117 times stronger activity than the parent peptide Si_1 (EC_{50} = 2.079 mM, extrapolated) and about 1.18 times weaker activity than caffeic acid (EC_{50} = 0.01513 mM). The lowest activity was observed for Si_{11}, with EC_{50} = 0.479 mM. This was about 32 times weaker than caffeic acid yet about 4.3 times stronger than Si_1. At 60 minutes only approximately 4 % of DPPH• was quenched by the parent peptide.

These results identify Si_{18} as the lead candidate approaching the caffeic acid benchmark under the tested conditions.

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