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Original Research Article

Antioxidant, Anti-Collagenase, Anti-Elastase and Anti-Tyrosinase Activities of an Aqueous *Cosmos caudatus* Kunth (Asteraceae) Leaf Extract

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ABSTRACT

Cosmos caudatus Kunth (Asteraceae) or wild cosmos is traditionally used as a culinary and medicinal herb. The leaves and shoots of the plant are reputed to promote a younger skin complexion if consumed regularly. However, research to support its potential use as a cosmeceutical is lacking. This study investigated the antioxidant, anti-collagenase, anti-elastase and anti-tyrosinase activities of *C. caudatus* leaf water extract (CCW) *in vitro* to scientifically evaluate its potential as a cosmeceutical. The antioxidant activity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, anti-collagenase and anti-elastase activity were measured using fluorescence assays and anti-tyrosinase assay was measured by colorimetric assay. The DPPH and ABTS assays showed CCW to have IC_{50} values of 163.6 and 57.2 µg/mL, respectively. Significant collagenase, elastase and tyrosinase inhibitions were observed with 51.3, 63.7 and 72.7% inhibition, respectively at 1000 µg/mL CCW treatment, compared with controls. Flavonol glycosides derived from quercetin and kaempferol, and flavone *C*-glycosides derived from apigenin were the main compounds identified in CCW using high resolution LC-UV-MS/MS. These results suggest the potential application of CCW as a cosmeceutical with antioxidant, anti-collagenase, anti-elastase, anti-tyrosinase activities.

Keywords: *Cosmos caudatus*, Antioxidant, Anti-Collagenase, Anti-Elastase, Anti-Tyrosinase.

Introduction

Cosmos caudatus Kunth (Asteraceae) is an annual herb originating from Latin and Central America, which later spread to the Asian region through the Philippines.[1] In Asian countries, such as Malaysia and Indonesia, the plant is believed to have anti-aging properties that can promote a younger skin complexion. *C. caudatus* is called "Ulam Raja" by the locals in Malaysia, which is literally translated as the King's Salad or "kenikir" in Indonesia. Their belief stems from the traditional practice of eating the plant parts (leaves and young shoots) as salad, either cooked or fresh, to defend against skin aging.[1,2] The pharmacological activities of the plant have been reported as antioxidant, anti-microbial, anti-fungal, anti-diabetic, anti-hypertensive, anti-inflammatory and osteoprotective.[3,4] Our extensive literature search indicates that there is a paucity of data on this plant species, especially its activities that could provide a scientific basis for its use against skin aging. With the current trend in the cosmetics industry to develop natural-based cosmetics, utilisation of *C. caudatus* leaf extracts as cosmetic ingredients based on scientific evidence, may add to the economic value to this plant species.

Antioxidants play a significant role in the management and intervention of skin aging because of the strong association of ageing with oxidative stress.[5–7] Current findings in the pathology of skin aging indicate that both intrinsic and extrinsic skin aging share similar fundamental pathways, which is the oxidative stress pathway.[8] Oxidative stress induced by UV radiation causes significant elevation of extracellular matrix (ECM) degradation enzymes, such as matrix-metalloproteinase (MMP) and elastase[9–13] and melanin producing tyrosinase,[14,15] which later leads to wrinkles, sagging and hyperpigmentation of the skin. In this study, the leaves of *C. caudatus* are investigated for antioxidant, anti-collagenase, anti-elastase and anti-tyrosinase activities, which are some of the key targets to reduce skin aging.

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Materials and Methods

Chemicals and reagents

The diphenyl-picryl hydrazine (DPPH) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), tyrosinase (T3824-25 KU), L-tyrosine, epigallocatechin-3-gallate (EGCG) of green tea origin and kojic acid were purchased from Sigma Aldrich, UK. 50 mM phosphate buffer, pH 6.8 was prepared from 0.1 M sodium dihydrogen phosphate (51 mL) and 0.1 M disodium phosphate dihydrate (49 mL). EnzChek® Gelatinase/Collagenase (E-12055) and Elastase (E-12056) Assay kits were purchased from ThermoFisher Scientific, UK.

Plant material

The plant material was purchased from Selangor, Malaysia. A voucher specimen was deposited in the Medicinal Plant Research Group Herbarium, Newcastle University, UK for future reference (Voucher no: CC/SEL-MY/NNAZ/001).

Extract preparation

The dried plant material (leaves) weighing approximately 100 g was powdered using an electric grinder. The plant powder was weighed

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(50 g) and infused with freshly boiled distilled water (500 mL) for 30 minutes. The water extract was chosen to mimic the traditional consumption as salad and organic solvents were avoided considering safer application as cosmeceuticals. The mixture was vacuumed-filtered, and the marc was discarded. The obtained filtrate was labelled as *Cosmos caudatus* water extract (CCW), freeze-dried, and finally stored at -20 °C. The freeze-dried extract was re-constituted in water prior to the experiments.

Antioxidant assays

DPPH assay: In a 96-well plate, Trolox (15 µL), plant samples (15 µL) and water or 70% ethanol (15 µL) as controls were added with 0.04 mg/mL DPPH working solution (285 µL) and properly mixed. The plate was incubated in the dark at 30°C for 30 min before a reading was taken at a wavelength of 517 nm using a microplate reader SpectraMax Plus[384].All experiments were performed in triplicates. Background absorbance was corrected by subtracting the absorbance value of a blank (water).

ABTS assay: In a 96-well plate, either Trolox standard solution (10 µL), plant sample (10 µL) or a solvent (10 µL) for control were thoroughly mixed with ABTS working solution* (290 µL) in the assay wells. The microplate was subsequently incubated in the dark at 37°C for 6 minutes. All experiments were performed in triplicate. The absorbance of the solution in each assay well was determined using a microplate reader (SpectraMax Plus[384], Molecular Device Corporation) at a wavelength of 734 nm. All experiments were performed in triplicates. Background absorbance was corrected by subtracting the absorbance value of a blank (water). *Working solution was a diluted solution (1/50 in PBS buffer) of preformed ABTS radical from 1:1 chemical reaction of 7 mM ABTS and 2.45 mM potassium persulfate.

Anti-collagenase assay

The protocol was followed according to the manufacturer's suggestions with some modifications. In a 96-well plate, 50 µL of collagenase (0.8 units/mL) was added to 50 µL of CCW (62.5-1000 µg/mL) or EGCG (positive control) and incubated in the dark for 15 min at 37°C. After pre-incubation, 100 µL of fluorescein labelled DQ gelatine (200 µg/mL) was added to the reaction well. The plate was further incubated for 120 min at 37 °C and fluorescence was measured every 15 min at Ex/Em of 485 ± 10/530 ± 15 nm emission using a FLUOstar Omega fluorescence microplate reader. Interval measurements were obtained to ensure the enzymes were active. The final concentration of the enzyme and substrate were 0.2 units/mL and 100 µg/mL, respectively. The results were expressed as percentage activity of CCW-treated-enzymes to untreated control.

Anti-elastase assay

This assay was performed according to the manufacturer's instructions with some modifications. In a 96-well plate, 100 µL of porcine pancreatic elastase (0.5 units/mL) was incubated with 50 µL of CCW (62.5-1000 µg/mL) or EGCG (positive control) in a 96-well plate in the dark at 25°C for 15 min. After preincubation, 50 µL of elastin solution (100 µg/mL) was added to each well to make a final reaction volume of 200 µL; the microplate was further incubated in the dark at 25°C for 120 min. Fluorescence were measured every 15 min at Ex/Em of 485 ± 10/530 ± 15 nm emission using FLUOstar Omega fluorescence microplate reader to ensure the enzyme was active. The final concentration of enzyme and substrate were 0.1 units/mL and 100 µg/mL, respectively. The results were expressed as percentage activity of CCW-treated enzymes compared with untreated control.

Anti-tyrosinase assay

The previously reported protocol was modified and optimized.[16,17] Briefly, CCW (62.5-1000 µg/mL) or kojic acid (positive control) (20 µL) were pre-incubated with 300 units/mL tyrosinase (20 µL) for 15 min at 25°C in a 96-wells plate. After incubation, 2 mM L-tyrosinase (160 µL) was added to obtain 200 µL of total volume reaction mixture and further incubated for 60 min. The final concentration of the enzyme and substrate were 30 units/mL and 1.6 mM, respectively. The enzyme activity was measured at 10 min intervals at 450 nm to ensure the enzyme was active throughout the incubation period. Percentage activity of CCW-treated-tyrosinase to untreated (control) was calculated and compared.

Percentage enzyme activity

Results were calculated based on the following equation:
Percentage enzyme activity (%) = Sample-treated enzyme /Untreated control x 100%.

Liquid chromatography-UV-serial mass spectrometry (LC-UV-MS/MS)

The methods for LC-UV-MS/MS were followed as in our previous publication.[18] The extract was reconstituted in the extraction solvent (water) at 10 mg/mL prior to chemical characterisation using LC–UV–MS/MS analysis. Analyses were performed on a Thermo Scientific system consisting of an 'Accela' U-HPLC unit with a photodiode array detector and an 'LTQ Orbitrap XL' mass spectrometer fitted with an electrospray source (Thermo Scientific, Waltham, MA, USA). Chromatography was performed by 5 µL sample injection onto a 150-mm x 3-mm, 3 µm Luna C-18 column (Phenomenex, Torrance, CA, USA) using 400µL/min mobile phase gradient of H_2O/CH_3OH/CH_3CN+1% HCOOH: 90:0:10 (0 min), 90:0:10 (5 min), 0:90:10 (60 min), 0:90:10 (65 min), 90:0:10 (67 min), 90:0:10 (70 min) followed by return to starting conditions and equilibration in starting conditions for 5 min before the next injection. The ESI source was operated with polarity switching and the mass spectrometer was set to record high resolution (30 k resolution) MS1 spectra (m/z 125–2000) in positive mode using the orbitrap and low resolution MS1 spectra (m/z 125– 2000) in negative mode and data dependent MS2 and MS3 spectra in both modes using the linear ion trap. Detected compounds were assigned by comparison of accurate mass data (based on ppm), and by available MS/MS data with reference to the published compound assignment system[19] and with supportive UV spectra.

Statistical Analysis

ANOVA was used to compare mean differences between treated and non-treated using Graphpad Prism Software with $p < 0.05$ considered as significant.

Results and Discussion

The antioxidant activities for CCW were evaluated using the DPPH and ABTS assays, and dose-dependent effects were observed in both assays (Figure 1). CCW was able to scavenge both preformed ABTS and DPPH radicals but the potency of the extract in both assays, expressed as IC_{50} values, was lower than of the Trolox standard (Table 1). In this experiment, CCW showed higher scavenging activity against ABTS free radicals compared with DPPH free radicals, where 100% inhibition was observed at 125-1000 µg/mL. The difference in CCW activities between the two assays may be caused by the structure difference of the preformed radicals.[20]

C. caudatus leaf extracts have been shown to have high antioxidant activities in different studies [21–25]. In one study, the antioxidant capacity of *C. caudatus* was reported to have the highest potency among 21 tropical plant extracts evaluated, and was comparable in potency to some synthetic antioxidants.[26] In this study, similar antioxidant activities were observed with CCW. Previous studies have revealed that the antioxidant activities associated with this species may be attributed to the flavonoids present, while vitamin C may also contribute to the observed antioxidant effects.[21] Flavonoids of the flavonol and flavone subclasses are widely documented to mediate antioxidant effects.[27–29]

In this study, high-resolution LC-UV-MS/MS analysis of CCW revealed the detection of compounds assigned as quercetin, kaempferol and apigenin glycosides assigned from their observed $[M + H]^+$ ions (Table 3), and supportive MS interpretation and UV

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spectra. Since flavonoids of these subclasses (flavonol glycosides and flavone *C*-glycosides) are associated with antioxidant effects,[27–30] it may be concluded that their detection in CCW may have contributed considerably to the antioxidant effects observed. We therefore confirm the antioxidant activity of an aqueous extract of *C. caudatus* leaf containing a range of flavonoid glycosides, and suggest this action can mechanistically complement other biological activities of CCW that are relevant to target skin ageing processes.

The anti-collagenase assay showed a dose-dependent collagenase inhibition by CCW. Significant reduction of collagenase activity was observed at 62.5, 125, 250, 500 and 1000 µg/mL with 5.5, 10.6, 17.2, 29.1 and 51.3 % inhibition, respectively (Figure 2A). Similarly, the EGCG positive control showed a dose-dependent inhibitory effect against collagenase activity, where significant inhibition at 31.6, 34.9, 41.1, 52.5, 96.4% was observed at 2.0-62.5 µg/mL (Figure 2B). The positive control EGCG had a lower IC_{50} value compared to CCW, as

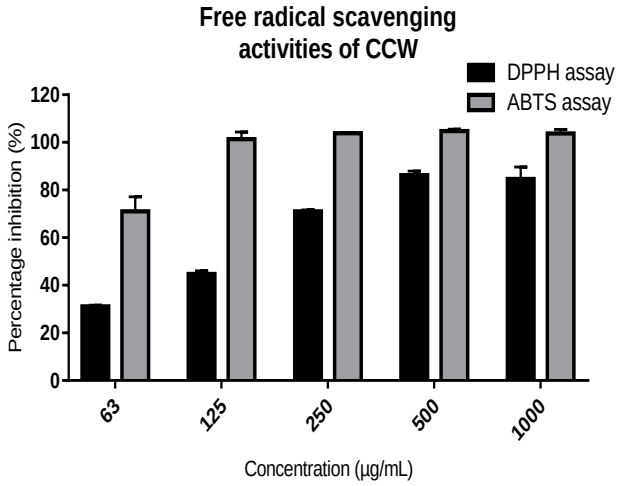

Figure 1: Dose-response of CCW in DPPH and ABTS assays data represent mean ± SD of triplicates.

Table 1: IC_{50} values for CCW and Trolox standard in DPPH and ABTS assays.

Assays	CCW IC_{50} (µg/mL)	Trolox IC_{50} (µg/mL)
DPPH	163.6	3.32
ABTS	57.2	6.51

Table 2: IC_{50} values for CCW, EGCG and Kojic acid standards in anti-collagenase, anti-elastase and anti-tyrosinase assays.

Assay	CCW IC_{50} (µg/mL)	EGCG/Kojic acid IC_{50} (µg/mL)
Anti-collagenase	926.8	13.2
Anti-elastase	758.0	487.0
Anti-tyrosinase	693.2	77.8

presented in Table 2, which may be due to the comparison of a single compound (EGCG) with CCW that contains a mixture of compounds.

A dose-dependent inhibitory effect was observed with treatment of CCW on elastase activity. Significant reduction in elastase activity was observed at 125, 250, 500 and 1000 µg/mL with 7.0, 19.0, 37.0 and 63.7% inhibition respectively (Figure 2C), compared with untreated control; whilst the positive control EGCG inhibited elastase activity by 39.0, 62.0, 82.0 and 90.0 % at 7.9 - 62.5 µg/mL (Figure 2D). The IC_{50} values of CCW and EGCG (Table 2) suggest that EGCG was a more potent elastase inhibitor than CCW.

A dose-dependent tyrosinase inhibitory effect was observed with treatment of CCW. Significant reduction in tyrosinase activity was observed at 250, 500 and 1000 µg/mL with 17.3, 37.1 and 72.7% inhibition, respectively, compared with the untreated control (Figure 2E). Kojic acid (positive control) inhibited tyrosinase by 13.9 and 63.0 % inhibition at 39 and 78 µg/mL, respectively (Figure 2F). Almost 100% tyrosinase inhibition was observed at 156, 313 and 625 µg/mL with the kojic acid treatment; thus, kojic acid was a more potent tyrosinase inhibitor than CCW (IC_{50} values are shown in Table 2).

The main compounds detected in the CCW extract by high resolution LC-UV-MS/MS in the positive ionisation mode were assigned as amino acids, flavonol glycosides, including those derived from quercetin and kaempferol, and flavone *C*-glycosides derived from apigenin (vicenin-2 and vitexin) (Table 3). Flavonol and flavone glycosides, and amino acids, have been shown to occur in this plant species previously [24,31]. Specifically, a previous study reported a range of quercetin glycosides to occur in leaves of this species,[31,32] which is in agreement with the compounds detected in CCW in this study.

The appearance of fine lines and wrinkles is the most common and prominent sign of skin ageing due to the degradation of the extracellular matrix (ECM) proteins such as collagen and elastin [33], which are regulated by matrix metalloproteinases (collagenases) and elastases. Furthermore, skin hyperpigmentation due to the excessive synthesis of melanin on the face and neck is aesthetically undesirable, and tyrosinase is the key enzyme that regulates the synthesis of melanin in the skin.[34] In this study, CCW had significant inhibitory effects against collagenase, elastase and tyrosinase (Figure 2 A-F). Therefore, the activities of CCW against collagenase, elastase and tyrosinase, combined with antioxidant effects, suggest its potential anti-aging properties for skin. An extensive literature search shows that these enzyme inhibitory activities have not previously been reported for *C. caudatus* extracts;[1,3,35] hence this study is the first report of these mechanisms to provide a scientific basis for the traditional and potential use of *C. caudatus* leaf extracts to target skin aging.

A range of other plant extracts have been investigated as potential collagenase and elastase inhibitors.[16,36–39] One of the known significant collagenase and elastase inhibitors are extracts of *Camellia sinensis* (L.) Kuntze such as white and green teas, which contain catechins,[36] hence the use of EGCG from *C. sinensis* as a positive control in the present study. Flavonoids including quercetin, kaempferol and apigenin have also been shown to significantly inhibit collagenase and elastase.[40–42] Thus, it may be hypothesized that the detection of flavonoid glycosides derived from quercetin, kaempferol and apigenin in CCW (Table 3) may have contributed to the collagenase and elastase inhibitory effects of CCW. A study by Sin and Kim (2005) on collagenase inhibition by different classes of flavonoids suggested that flavonols were more potent inhibitors compared with other flavonoids due to their C-3-hydroxyl group; a similar mechanism has been proposed by Pientaweeratch *et al.* (2016), as shown by collagenase inhibition by a *Phyllanthus emblica* L. (amla) extract. The diversity of flavonol glycosides in CCW was greater than the other flavonoid classes detected, suggesting this compound class contributed to the elastase inhibitory activity observed, in agreement with previous findings of Sin and Kim (2005). MMPs, including collagenases are a group of zinc-dependent proteinases,[44] and flavonoids, including the flavonols quercetin and kaempferol, are known to have the ability to chelate metal ions, thus may also bind to the Zn ion at the collagenase active site and prevent the activation of the enzymes by the substrates.[45]

Kaempferol (3,4',5,7-tetrahydroxyflavone), quercetin (3,3',4',5,7-pentahydroxyflavone) and myricetin (3,3',4',5,5',7-hexahydroxyflavone) have also been shown to inhibit elastase dose-dependently due to the presence of the catechol group at the B-ring that determined the potency of the inhibition against elastase.[46] In CCW, the flavonols detected were assigned as the mono-glycosides of quercetin (primarily) and kaempferol, thus retaining the high degree of hydroxylation in the chemical structures that may explain the elastase

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inhibition observed. It is also suggested that these compounds inhibited elastase by acting as complexing or precipitating agents [47,48], as compared to catechin and EGCG from green tea via non-covalent bonding.[49,50] Similarly, quercetin and kaempferol have also been shown to significantly inhibit tyrosinase activity [34], thus the detection of quercetin and kaempferol glycosides may have contributed to the inhibitory effects of CCW against tyrosinase. Quercetin has been shown to be a competitive tyrosinase inhibitor able to inhibit melanin production dose-dependently in B16 melanoma cells.[51,52] Although some conflicting results have been reported on the role of quercetin in melanogenesis,[53] it is postulated that the inhibitory activity of quercetin is strictly dependent on the concentration tested, where the inhibitory activity against tyrosinase would then be observed as shown by Yang *et al.* (2011).

In the present study, the flavonols detected in CCW were in the mono-glycoside form, thus substitution of hydroxyl groups in quercetin or kaempferol by sugar moieties may have implications on the structure-activity relationships in enzyme inhibition. The presence of glycoside substituents in a flavonoid structure has been suggested to affect the coplanarity of the structure and delocalization of the electrons, as well as enhancement of polarity and increasing steric hindrance, that may contribute the observed activity [55,56]. Increasing the degree of glycosylation, and the position and structure of the saccharide may also significantly influence the structure-activity relationship for enzyme inhibition.[55,57] For example, an enhancement of enzyme inhibition was shown by puerarin, a glycosylated daidzein, in comparison to the aglycone daidzein [58]. These previous studies are therefore supportive evidence for the role of flavonoid mono-glycosides, as detected in CCW, for enzyme inhibition relevant to targeting mechanisms involved in skin ageing.

Table 3: Compounds assigned from LC-UV-MS/MS analysis of CCW

No.	Assigned compound[#] (or isomer)	Retention time (min)	Molecular formula	(*m/z*)	Ion	CCW ppm[#]
1.	Phenylalanine	2.9	$C_9H_{11}NO_2$	166.0868	$[M + H]^+$	3.160
2.	Alanylleucine, leucylalanine or isoleucylalanine	3.0	$C_9H_{18}N_2O_3$	203.1397	$[M + H]^+$	3.451
3.	Phenylalanylalanine or alanylphenylalanin	4.0	$C_{12}H_{16}N_2O_3$	237.1244	$[M + H]^+$	4.180
4.	Tryptophan	4.3	$C_{11}H_{12}N_2O_2$	205.0978	$[M + H]^+$	2.759
5.	Leucylvaline or Valylleucine	4.4	$C_{11}H_{22}N_2O_3$	231.1710	$[M + H]^+$	3.119
6.	Glutamylleucine, glutamylisoleucine or leucylglutamic acid	5.5	$C_{11}H_{20}N_2O_5$	261.1452	$[M + H]^+$	2.802
7.	Glutamylphenylalanine or phenylalanylglutamic acid	6.9	$C_{14}H_{18}N_2O_5$	295.1298	$[M + H]^+$	3.191
8.	Leucylleucine or leucylisoleucine	7.4	$C_{12}H_{24}N_2O_3$	245.1868	$[M + H]^+$	3.266
9.	Leucylleucine or leucylisoleucine	9.3	$C_{12}H_{24}N_2O_3$	245.1867	$[M + H]^+$	2.818
10.	Vicenin-2	12.5	$C_{27}H_{30}O_{15}$	595.1669	$[M + H]^+$	1.888
11.	Vitexin	18.6	$C_{21}H_{20}O_{10}$	433.1147	$[M + H]^+$	4.772
12.	Quercetin hexoside	19.7	$C_{21}H_{20}O_{12}$	465.1043	$[M + H]^+$	3.478
13.	Quercetin hexoside	20.3	$C_{21}H_{20}O_{12}$	465.104	$[M + H]^+$	4.338
14.	Quercetin pentoside	21.3	$C_{20}H_{18}O_{11}$	435.0942	$[M + H]^+$	4.510
15.	Quercetin pentoside	21.9	$C_{20}H_{18}O_{11}$	435.0937	$[M + H]^+$	4.441
16.	Quercetin pentoside	22.7	$C_{20}H_{18}O_{11}$	435.0942	$[M + H]^+$	Nd
17.	Quercetin rhamnoside	23.6	$C_{21}H_{20}O_{11}$	449.1092	$[M + H]^+$	2.944
18.	Di-*O*-Isopropylidene-*C*-methyl-*O*-methyl-hexose	25.2	$C_{14}H_{24}O_6$	289.1662	$[M + H]^+$	5.551
19.	Kaempferol rhamnoside	27.0	$C_{21}H_{20}O_{10}$	433.1145	$[M + H]^+$	4.079
20.	Heptanol pentosyl-hexoside	27.4	$C_{18}H_{34}O_{10}$	428.2515	$[M + NH4]^+$	5.785
21.	Di-*O*-Acetyl-di-*O*-isopropylidene-*glycero-hexo*-heptose	28.4	$C_{17}H_{26}O_9$	392.1932	$[M + NH4]^+$	4.263
22.	1-Octen-3-ol pentosyl-hexoside	29.8	$C_{19}H_{34}O_{10}$	440.2509	$[M + NH4]^+$	4.514

All compounds assigned by comparison of accurate mass data (based on ppm[#]), and by interpretation of available MS/MS and/or UV spectra.

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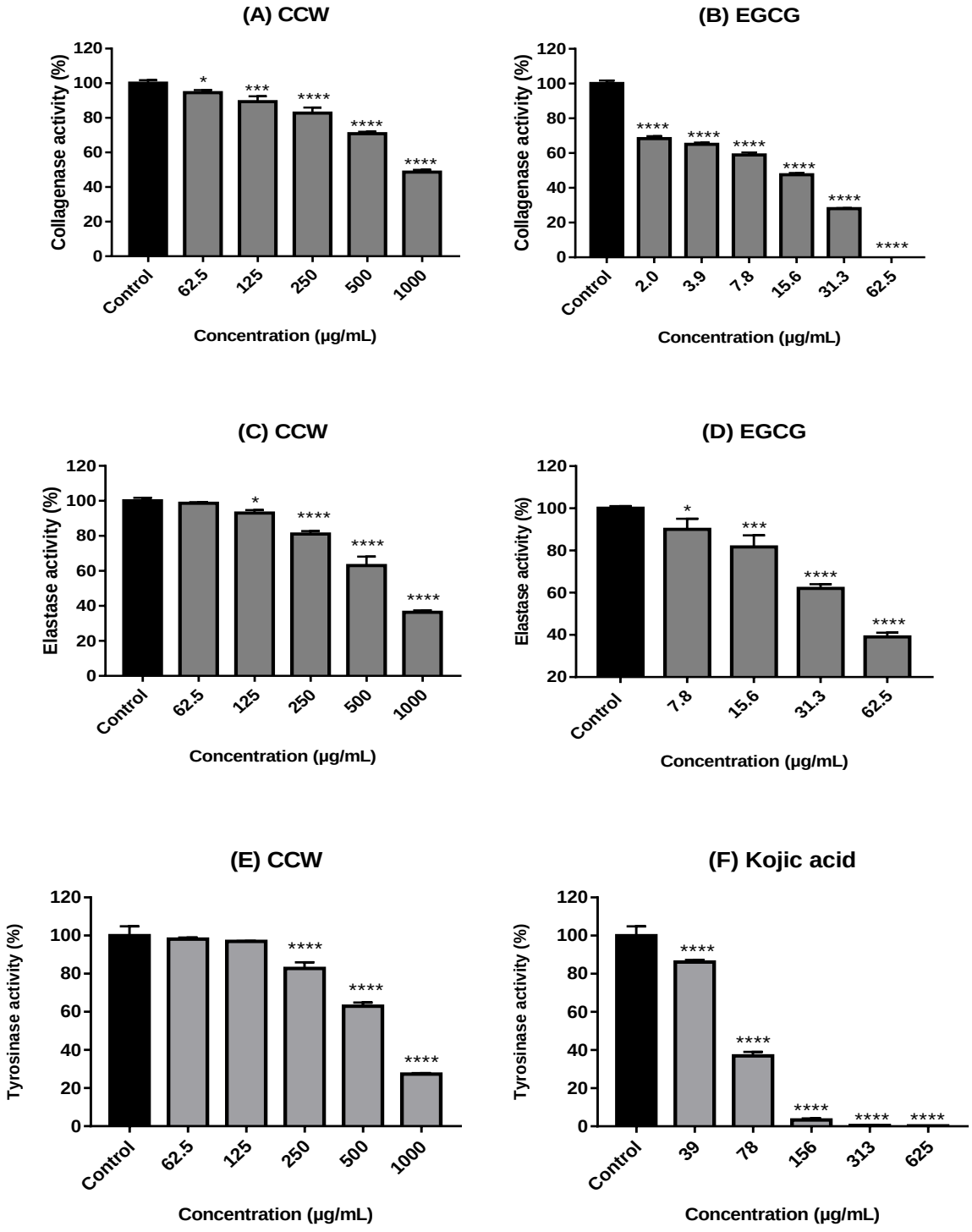

Figure 2: Anti-collagenase activities of CCW and EGCG (**A** and **B**), anti-elastase activities of CCW and EGCG (**C** and **D**) and anti-tyrosinase activities of CCW and kojic acid (**E** and **F**). Data are mean ± SEM of triplicates. *$p < 0.5$ ***$p < 0.001$ and ****$p < 0.0001$ represent significant mean differences compared with control (untreated enzymes).

Conclusion

This study demonstrated that an aqueous leaf extract of *C. caudatus* that contains flavonol glycosides derived from quercetin and kaempferol, and flavone *C*-glycosides derived from apigenin, could inhibit collagenase, elastase and tyrosinase. These results provide a scientific basis for the potential use of *C. caudatus* leaf extract as an anti-aging agent for skin, with antioxidant, anti-collagenase, anti-elastase and anti-tyrosinase properties; and justifies further evaluation of the extract and its constituents for their ability to protect against skin aging. Additionally, this study provides the first scientific evidence for the traditional use of *C. caudatus* leaf as a defence against skin aging.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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