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*Corresponding author: Md Shamsuddin Sultan Khan, School of Pharmaceutical Sciences, University of Science Malaysia, Penang, Malaysia
E-mail: jupitex@gmail.com

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PHARMACOLOGY | SHORT COMMUNICATION

Effect of crystallization of caffeic acid enhanced stability and dual biological efficacy

Md Shamsuddin Sultan Khan^{1*}, Muhammad Adnan Iqbal² and Amin Malik Shah Abdul Majid¹

Abstract: Caffeic acid (CA) is a well-known natural compound used for both health supplement and metabolic diseases. In the present study, single, easy, and quick isolation procedure of caffeic acid was described from rosmarinic acid. A quick crystal formation also was described for highly hygroscopic CA which was spontaneous and stable with lower hygroscopic nature. In addition, the produced crystal CA was highly stable and showed neuroprotective activity with $<6 \mu\text{g/ml}$ in R28 cell line and cytotoxic activity with $>100 \mu\text{g/ml}$ in U87 MG which revealed its dual biological effect for the treatment of Alzheimer's disease and cancer, respectively.

Subjects: Analysis & Pharmaceutical Quality; Analytical Chemistry; Cancer; Natural Products; Organic Chemistry; Pharmacology

Keywords: caffeic acid; rosmarinic acid; neuroprotective activity; cytotoxicity; crystallography; cancer; Alzheimer's diseases

1. Introduction

Caffeic acid (CA) is an interesting small molecule due to its chemical stability and antioxidant power (Chen & Ho, 1997; Gulcin, 2006; Hong, Yang, Nam, Koo, & Lee, 2015). It is a potent molecule which started a rational-based drug discovery for wide range of therapeutic application (Rezg et al., 2015; Rosendahl et al., 2015). Chemical structure of caffeic acid is delicate to drive any kind of desired scaffold hope. In our lab, we routinely check the phytochemicals and computationally design their newer derivatives which ultimately may become suitable candidates for the treatment of Alzheimer's disease and metabolic disorder like cancer. Earlier studies on caffeic acid showed its mild stimulant effect (Liszt, Eder, Wendelin, & Somoza, 2015; Shin et al., 2015; Toth et al., 2015). The past studies also showed its cytoprotective effect (Lee et al., 2015; Nunes et al., 2015), antiangiogenic effect, and moderate toxicity (Akyol et al., 2015; Kosova, Kurt, Olmez, Tuđlu, & Ari, 2015) that can be affected due to the stability of the compounds. This earlier observation postulates that caffeic acid can show

ABOUT THE AUTHORS

The authors are working to find out the differential efficacy of a drug and that solution. This kind of problem is seen during the early development of the drug in clinical case and thereby their failure of approval and marketing. Here, authors are trying to discuss the physical development of a candidate drug which might at least reduce risk of potency of a drug in different clinical cases.

PUBLIC INTEREST STATEMENT

The emerging growing interest to design the formulation in improving drug's shelf life is becoming viable to eradicate their differential efficacy. In the current study, a kind of viable natural solution of increasing shelf-life of the chemical compound and/or drug was described that showed worth even the smallest risk of differential efficacy in clinical set. The formulation scientist's genuine interest should look after the drug's behavior in different states of them under clinical use.

its dual biological efficacy due to half-life, solubility, stability, and physicochemical properties of the compound. Caffeic acid is highly hygroscopic and degrades at room temperature and in the presence of light. To determine the stability impact of the CA in biological activity is essential to assess the therapeutic efficacy. There is no evidence yet to explain the dual biological efficacy of the CA yet. The hygroscopic nature has greater impact for the CA bioactivity which might lead to the dual efficacy of this compound. In the present study, our objective was to stabilize the CA using natural ways and to find the reason of the dual efficacy of the same compound.

2. Experimental

2.1. Crystallography

All reagents were purchased from Fluka™, Aldrich™, and JT Baker™, USA. All solvents were used as received without further purification. The isolated caffeic acid was dissolved by adding methanol dropwise and kept under vacuum hood for two weeks at room temperature. After slow evaporation, faint yellow microcrystals were obtained. The crystals were filtered off and washed by cold methanol at room temperature. The microcrystals solution of Dimethylformamide (DMF) was slowly evaporated to determine the crystal's chemical structure suitable for X-ray diffraction studies. Crystal data were obtained on a Bruker SMART APEXII CCD area-detector diffractometer using graphite monochromated MoK α radiation ($\lambda = 0.71073 \text{ \AA}$) at 100 K. A crystal with dimension $9 \times 8 \times 31 \text{ mm}$ was used. The data were collected and reduced using Cambridge online crystallography program and Crystallo Chemcraft win version 1.8. The structure of all compounds was solved using the Crystallo Chemcraft win version 1.8 program package, and refined using the Crystallo Chemcraft win version 1.8 program package. The molecular graphics were created using Crystallo Chemcraft win version 1.8.

2.2. Cell culture and reagents

The rat retinal cell line R28 was purchased from Kerafast, USA, and human glioblastoma cell line U87-MG was bought from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified eagle medium (DMEM) (GIBCO, USA) supplemented with 10% heat inactivated fetal bovine serum (HIFBS) (GIBCO), 100 units/mL penicillin-streptomycin (GIBCO), and 2 mM glutamine (GIBCO) in a humidified incubator with 5% CO₂ at 37°C. Rosmarinic acid (RA) was purchased from Sigma (Germany). MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), trypsin, and phosphate-buffered saline (PBS) were purchased from Sigma. Matrigel matrix (10 mg/mL) was obtained from BD Bioscience (USA). About 10 mg of powdered RA (96% purity) was dissolved in sterile, cell-cultured tested DMSO (Sigma, USA) to prepare a 10 mg/mL stock solution, which was stored in a moisture controlled environment at RT. Other chemicals used were of analytical grade.

2.3. In vitro cytotoxicity and cytoprotection

The effects of CA on R28 cell and U87MG toxicity were determined using the MTT at 0.004 $\mu\text{g/ml}$ (i.e. 20 μl from 5 mg/ml stock solution) cell proliferation assay. Briefly, R28 and U87MG cells (70% confluent) were seeded in 96-well plates and cultured overnight followed by treatment with different concentrations of CA (6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g/ml}$). Cells were incubated for 48 h at 37°C and 5% CO₂, and cell proliferation and cytotoxicity were calculated using a regression equation (below). After 48 h of treatment, MTT solution (20 μl) was added to each well and the mixture was re-incubated for 4 h. Absorbance of the samples then was read at 490–570 nm, and percentage proliferation and cytotoxicity (percent inhibition (i.e. IC₅₀)) were calculated as follows:

$$\% \text{ Inhibition} = 1 - \frac{\text{Absorbance of treated}}{\text{Absorbance of untreated}} \times 100$$

The % inhibition was plotted against the concentration tested using Microsoft Excel, and the IC_{50} was calculated using the non-linear regression equation. Effect of cytoprotective activity of the crystal CA was determined using serum deprivation assay. Cells were treated with and without serum and after 48 h of treatment and MTT assay was conducted as discussed above. Percent proliferation was determined according to earlier study of Khan et al. (2016).

2.4. Stability study

The thermal, chemical, biological, and environmental stability of the CA was determined according to earlier described study by Khan et al. (2016). In brief, thermal stability was determined by HPLC after treating the caffeic acid (50 mg of powder and crystal) at 60°C in the oven for 48 h. For chemical stability study, caffeic acid was treated with LiOH and quantity of caffeic acid was determined by mass balance and HPLC. Conversely, HCl and Krebs-Henseleit bicarbonate buffer (pH 7.4) treated caffeic acid at 37°C for 2 h was quantified by HPLC peak and time. To determine the biological stability, caffeic acid (10 mM of powder and crystal) was treated by human serum which was then deproteinized using 500 μ L of acetonitrile containing 0.1% trifluoroacetic acid and filtered off to determine the quantity in HPLC. The HPLC method was validated according to earlier described method by Khan et al. (2016).

3. Results and discussion

Keeping in view the literature information of CA, we successfully hydrolyzed the rosmarinic acid (Scheme 1) in the presence of acid as catalysis (polyphosphoric acid) and obtained crystals of CA (He, Li, & You, 2015). The reaction was carried out by mixing 1 equivalent of RA with 15 equivalents of H_3PO_4 to get a thick paste. The paste was heated to 150°C and poured into a large volume of stirring cold water. The solution was then neutralized by sodium hydroxide and the white precipitates were collected. Recrystallization by methanol provided colorless crystals of CA. The melting point of the crystals was found to be more than 300°C. The expected component β -aryllactic acid (β -AA) could not be isolated from the reaction mixture (Scheme 1). The crystals of CA so obtained were exposed to X-ray crystallographic machine. The molecular structure of CA along with the atomic numbering scheme and its packing in the crystal lattice are given in Figure 1(A) and (B).

The summary of the crystal data of CA is provided in the Table 1. Table S1 shows the selected bond lengths (\AA) and angles ($^\circ$), see supplementary file. The compound crystallizes into a monoclinic crystal system with space group P21/n. According to the molecular structure obtained from X-ray crystallographic technique, the CA contains a carboxylic group connected to a benzene ring containing two hydroxy groups through an alkyl chain (C6–C7–C8), see Figure 1(A). The molecular structure of the CA can be divided into two planar moieties that are connected by a single C–C bond. The first plane, the pyrocatechol plane, includes OH, OH, and benzene ring, whereas the second plane, the (E)-but-2-enoic acid plane, includes CO, OH, and C7–C8. Dihedral angle between these two planes is

Scheme 1. Isolation of caffeic acid (CA) from rosmarinic acid (RA).

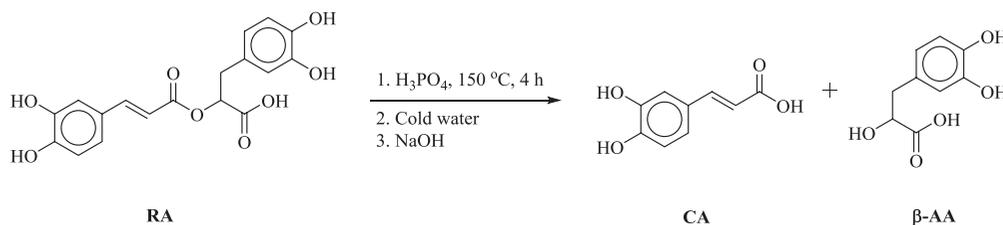
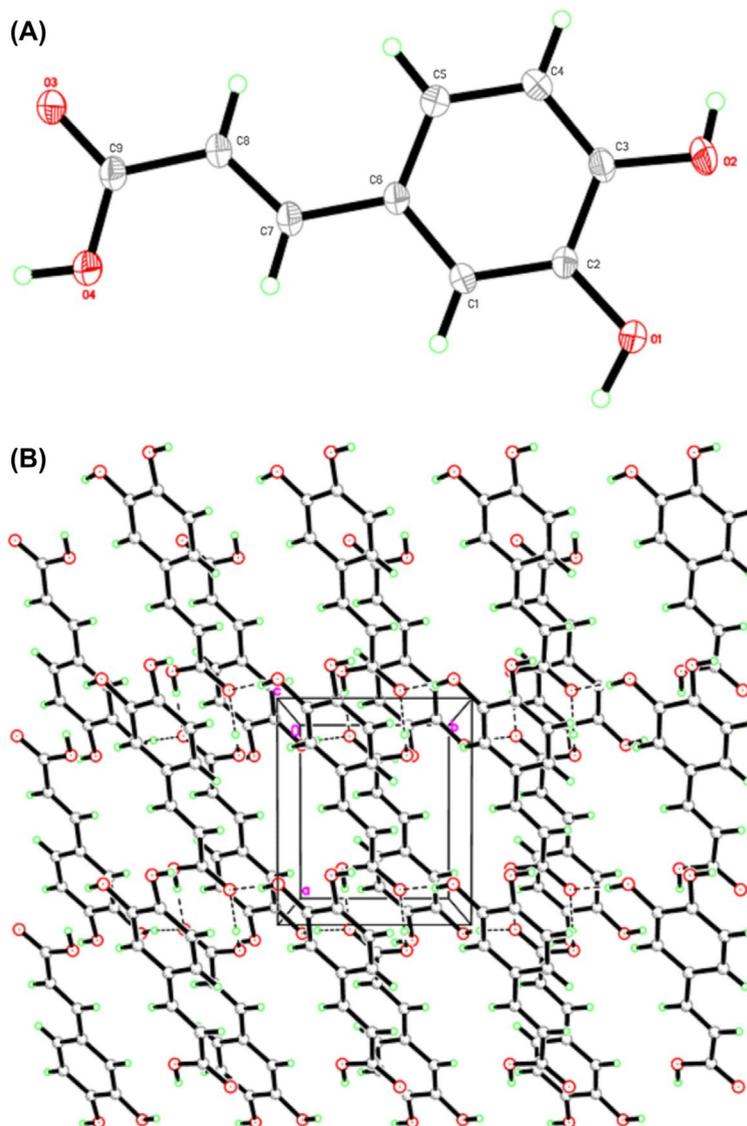


Figure 1. (A) The molecular structure of caffeic acid (CA) draw at 50% probability (B) shows the molecular packing.

Note: Crystal CCDC Depository CRM: 0001000261615, CCDC 1433127.



122.55(3)°. In CA, the C7–C8–benzene torsion angle is 178.94(15)°. In the crystal lattice, the packing of the molecules is stabilized by intermolecular hydrogen bonds. Further stabilization is provided by C–H– π and π – π interactions.

The ring and (E)-but-2-enoic acid are not in the same plane. The reason behind this is the packing effects where the molecules have low π – π stacking interactions. Figure 1(B) illustrates the crystal packing pattern, which shows that the molecules are connected through the C–O–H bonding in a three-dimensional network. The details of bond angles, lengths and torsions were presented in the Figure 3.

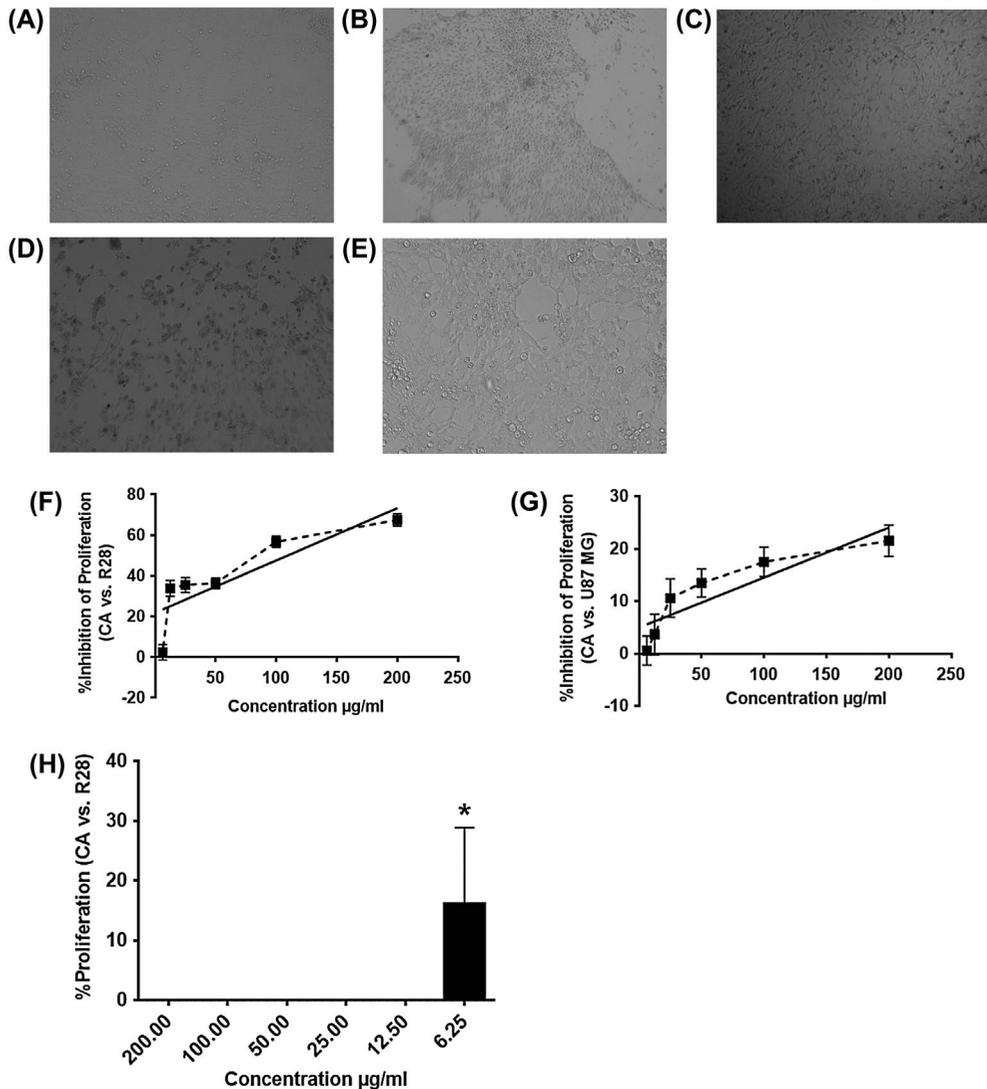
Table 1. Crystal data and structure refinement parameters for CA

Compound	CA
Empirical formula	C ₉ H ₈ O ₄
Formula weight	180.15
Temperature (K)	294 K
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	P 2 ₁ /n
<i>Unit cell dimensions</i>	
<i>a</i> (Å)	6.7154(5)
<i>b</i> (Å)	5.7725(4)
<i>c</i> (Å)	21.1336(16)
α (°)	90
β (°)	93.9107(11)
γ (°)	90
Volume (Å ³)	817.33(10)
Z	4
Calculated density (D _x , g cm ⁻³)	1.464
Radiation type λ (Å)	M _o K α
<i>F</i> (0 0 0)	376.0
Crystal size (mm) (<i>h</i> , <i>k</i> , <i>l</i> _{max})	9 × 8 × 31
Crystal color	Faint yellow
Scan range θ (°)(max)	31.622
Absorption coefficient (μ) (mm ⁻¹)	0.117
Maximum and minimum transmission	0.986 and 0.950
Goodness-of-fit on <i>F</i> ²	0.995
Data/restraints/parameters	2736/1.119/130
Final <i>R</i> indices [<i>I</i> > 2 σ (<i>I</i>)]	<i>R</i> ₁ = 0.0629, <i>wR</i> ₂ = 0.1861

The crystals of CA were evaluated for the safety in rat neurosensory retinal (R28), and human glioblastoma U87MG cells by inducing serum insult. The R28 retinal precursor cell line is useful to determine the retinal cell behavior including differentiation, neuroprotection, cytotoxicity, light stimulation, retinal gene expression, and neuronal function. R28 cell line is nontumorigenic and the toxic effect on this cell line refers to the injury and inflammation of retina. R28 cells and U87MG cells were treated with 200 μ g/mL to 6.25 μ g/mL of CA for 48 h. Cell viability was measured using MTT assay. The cell viabilities of R28 cells, and U87MG cells treated with CA were significantly different ($p > 0.05$) from that of untreated controls. The IC₅₀ values were found in R28 as 109.53 μ g/ml (Figure 2(A), (B), and (F)) and in U87MG as 532.84 μ g/ml (Figure 2(C), (D), and (G)). There was significant difference ($p > 0.05$) between viabilities of R28 and U87 MG cells treated with CA. This report suggests that CA, at the given concentrations or above the dose normally used in clinical practice, is toxic to rat neurosensory retinal, and human glioblastoma cells *in vitro*. The reason for this dual effect of crystal CA may be due to stability and solubility parameters. The effect of crystal CA with 6.25 μ g/mL had obvious cytoprotective effect which was slightly different with the CA contained moisture (Figure 2(E) and (H)). In each

Figure 2. *In vitro* toxic effects of CA in the R28 cell lines (A, B) and U87 MG cells (C, D). The IC₅₀ of CA was 109.52 µg/ml (F) and 532.83 µg/ml (G) for R28 and U87 MG cells, respectively. The R28 cells were not affected and proliferated at lowest dose (6.25 µg/ml) of caffeic acid (E, H) in serum insult assay.

Note: The dose dependent effects of CA was plotted and IC₅₀ was determined using non-linear regression analysis.



case of stability study, caffeic acid was determined by HPLC before and after treatment of chemical, thermal, biological, and environmental factors. The elution time of caffeic acid was 3–5 min that revealed the affected stability for its solid composition of powder and crystal form. The crystal form of the caffeic acid was more stable than powdered form that the elution time was constant for crystal but it was deviated with multiple peaks at different elution time for powdered form which might be the reason of moisture content (Figures S1 and S2).

Supplementary material

The supplementary material for this paper is available online at <http://dx.doi.org/10.1080/23312025.2016.1243460>.

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Author details

Md Shamsuddin Sultan Khan¹

E-mail: jupitex@gmail.com

Muhammad Adnan Iqbal²

E-mail: adnan_chem38@yahoo.com

ORCID ID: <http://orcid.org/0000-0001-6241-7547>

Amin Malik Shah Abdul Majid¹

E-mail: aminmalikshah@gmail.com

¹ School of Pharmaceutical Sciences, University of Science Malaysia, Penang, Malaysia.

² School of Chemical Sciences, University of Science Malaysia, Penang, Malaysia.

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