Original Article

Angiotensin-converting Enzyme and Renin Inhibition Activities, Antioxidant Properties, Phenolic and Flavonoid Contents of *Cuphea ignea* A. DC.

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Abstract

Cuphea ignea A. DC. is an ornamental plant belonging to family Lythraceae distributed in the tropics and temperate zones. Some *Cuphea* members are traditionally used in treatment of hypertension. The aim of this research was to study the antihypertensive activity of the plant by applying a bio-guided fractionation scheme of the leaves, stems, and flowers using *in vitro* angiotensin-converting enzyme (ACE) and renin inhibition assays to identify the active organ and its respective active fractions. The aqueous extract and the *n*-butanol fraction of the leaves showed the highest ACE inhibition activity with IC_{50} values 0.491 and 0.084 mg/mL, respectively. The renin inhibition activity showed that the alcoholic extract of the leaves had the highest percentage of inhibition (94.82%). Meanwhile, the methylene chloride fraction of the stems showed the highest renin inhibition activity (98.14%) followed by the ethyl acetate fraction of the leaves (93.09%). The antioxidant activity was tested using the 2,2-diphenyl-1-picrylhydrazyl and oxygen radical absorbance activity assays. Correlation studies of the antioxidant activity showed moderate correlation with the phenolic content and strong correlation with the flavonoid content. The renin inhibition activity showed significant positive correlation with the phenolic and flavonoid contents with *r* values (0.777 and 0.629, respectively).

Keywords: *Antihypertensive, angiotensin, correlation, Cuphea, Lythraceae*

Introduction

Hypertension is one of most dominant diseases worldwide, which can cause about 12.8% of the total deaths.[1] Hypertension can be because of the activity of renin angiotensin aldosterone system (RAAS).[2] Inhibition of angiotensin I-converting enzyme (ACE) and renin is one of the effective strategies in exploring new antihypertensive remedies.[3,4] Previous studies have shown that certain flavonoids show a capacity to inhibit different zinc metalloproteinases including ACE.[5,6] Also, it was reported that the phenolic compounds including flavonoids inhibit ACE via interaction with the zinc ion in the active site where the number of hydroxyl groups on the benzene ring plays an important role in the ACE inhibition.[7,8] The reactive oxygen species (ROS) plays a role in cardiovascular diseases.[9] As a result, it is beneficial for an antihypertensive drug to have antioxidant potential.[10] *Cuphea ignea* A. DC. is an ornamental plant belonging to family Lythraceae and distributed in the tropic and temperate zones.[11] Some *Cuphea* members were traditionally used in treatment of hypertension, for example, aerial parts of *Cuphea calophylla* Cham. & Schltdl. and *Cuphea aperta* Koehne.[12,13] The ethanolic extract of the aerial parts of *Cuphea carthagenensis* (Jacq.) J.F.Macbr. showed ACE inhibition activity.[14] Nothing was reported regarding the antihypertensive activity of *Cuphea ignea* A. DC. As a part of our ongoing research for a natural therapeutic approach for treatment of high blood pressure, *in vitro* screening has been conducted to evaluate the ACE and renin inhibition activities together with the antioxidant activity of the different extracts of *Cuphea ignea* A. DC. These activities were studied in relation to the plant's phenolic and flavonoidal contents.

Material and Methods

Plant material

Samples of *Cuphea ignea* A. DC. used in this study were obtained in the flowering stage in May 2015 from El Kanater El Khayreya, Egypt and were kindly identified by Dr. Mohamed El-Gebali, Senior Botanist at El-Orman Botanic Garden, Egypt. A voucher specimen (No. 4-5-2016-1) was kept in the Herbarium of

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Chemicals

All chemical reagents and extraction solvents were of analytical grade and all analysis solvents were of high-performance liquid chromatography grade and were obtained from the local market. ACE (from rabbit lung), histidine-L-hippuryl-L-leucine-chloride (HHL), renin (human recombinant) and Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(DABCYL)-Arg, lisinopril, aliskiren, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, gallic acid, quercetin, aluminum chloride, ascorbic acid, fluorescein, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), and methanol were all purchased from Sigma-Aldrich, Germany. Borate saline buffer components (boric acid, KCL, and NaOH), dimethyl sulfoxide (DMSO), tris HCl, and NaCl were purchased from Merck, Darmstadt, Germany.

Preparation of extracts

The alcoholic extracts

The air-dried powdered leaves (80 g), stems (240 g), and flowers (120 g) were separately extracted with 95% ethanol by cold maceration till exhaustion (6 × 500 mL, 5 × 700 mL, and 5 × 300 mL, respectively). The alcoholic extracts were separately evaporated under reduced pressure till dryness at a temperature not exceeding 50°C to give 9 g, 9.5 g, and 5 g of dry residues of the leaves, stems, and flowers, respectively. Different dilutions of the extracts were prepared in distilled water containing few drops of Tween 80 and saved for the biological study.

The aqueous extracts

The air-dried powdered leaves and stems (100 g each) were separately macerated in boiling distilled water for 1 h and then filtered (4 × 500 mL). The aqueous filtrates were separately lyophilized to give 19 g and 11.6 g of dry residues of the leaves and stems, respectively.

Fractionation of the alcoholic extracts

The alcoholic extracts of the leaves (8 g) and the stems (8.5 g) were separately suspended in water (40 mL) and successively fractionated with petroleum ether (4 × 60 mL), methylene chloride (6 × 50 mL), ethyl acetate (5 × 50 mL), and *n*-butanol saturated with water (5 × 60 mL). Collective fractions were evaporated under vacuum to obtain dry residues weighing 2.1 g, 0.38 g, 1.5 g, and 2.9 g for the petroleum ether, methylene chloride, ethyl acetate, and *n*-butanol fractions of the leaves, respectively, and 1.46 g, 0.24 g, 0.53 g, and 3 g for the stems, respectively.

Quantitative estimation of the total phenolic content

Spectrophotometric determination of the total phenolic content of the alcoholic and aqueous extracts was carried out using the Folin–Ciocalteu colorimetric method.[15] A stock solution of gallic acid (1 mg/mL) and a standard calibration curve was established using serial dilutions ranging from 20 to 280 µg/mL in methanol. The total alcoholic and aqueous extracts were dissolved in methanol in concentration 1 mg/mL and 1 mL of each extract was tested. The absorbance was measured at 760 nm using a spectrophotometer (Shimadzu UV-1650PC, Duisburg, Germany) against a blank (methanol instead of the test solution). Three replicates were carried out for each concentration.

Quantitative estimation of the total flavonoid content

The method was based on measuring the intensity of the color developed from complexing aluminum chloride reagent with flavonoids.[16] The percentage of flavonoids in the alcoholic and aqueous extracts was calculated as quercetin equivalent using a preestablished standard calibration curve of concentrations 5–140 µg/mL. The total alcoholic and aqueous extract residues were dissolved in methanol in a concentration 1 mg/mL. The absorbance measured at 415 nm. All experiments were performed three times.

Determination of the antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl assay

The antioxidant activity was evaluated using the quantitative DPPH assay[17] using ascorbic acid, which was used as positive control. Different dilutions of each sample were prepared in triplicates and blank samples were prepared using methanol. After incubation of the mixture at 37°C for 30 min in the dark, the absorbance was measured at 517 nm. The percentage of inhibition of DPPH (I %) ± standard deviation (SD) (n = 3) was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{A.control} - (\text{A.sample} - \text{A.blank})}{\text{A.control}} \times 100$$,where

A.control, A.blank, and A.sample are the absorbances of the control, blank, and the extract, respectively. The concentration in mg/mL causing 50% inhibition of the free radical (IC_{50} ± SD [n = 3]) was determined.

Oxygen radical absorbance capacity assay

The evaluation of the oxygen radical absorbance activity (ORAC) was performed as previously reported.[17] The antioxidant potential of the extracts was measured by calculating the fluorescence decay time of fluorescein resulted from AAPH as compared with trolox. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of the fluorochrome.

Evaluation of *in vitro* antihypertensive activity

Angiotensin-converting enzyme inhibition assay

The ACE inhibition potential of the different plant extracts and fractions was tested using the method of Balasuriya and Rupasinghe.[18] with slight modifications. The method depends on formation of His-Leu by the cleavage of HHL in the presence of ACE, which is measured using the fluorescence method. All the plant extracts and fractions were dissolved in methanol (100 mg/mL). Serial dilutions were prepared for each test solution (0.1–100 mg/mL) and for each fraction (0.01–100 mg/mL). Lisinopril was used as a reference drug in concentration ranging from 0.0001 to 0.1 mg/mL.

The test solutions were prepared by adding 40-µL enzyme solution (2-mU ACE prepared in 0.1-M Na borate buffer) to 20 µL of each tested dilution of each sample and then incubated at 37°C for 10 min, followed by addition of 40-µL HHL substrate (0.8 mM/L). The test solutions were all incubated at 37°C for 1 h and 60-µL 0.5-M sodium hydroxide was then added to stop the reaction. Blank solutions were prepared for each sample by adding buffer solution instead of the enzyme solution. Control solutions was prepared using methanol instead of the sample. Triplicates were run for each sample. Experiments were set in 96-well microplates. The fluorescence was measured at excitation (360 nm) and emission wavelengths (500 nm) using the FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). The percentages of inhibition (% I ± SD) were calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Fl.control} - (\text{Fl.sample} - \text{Fl.blank})}{\text{Fl.control}} \times 100 \text{, where}$$

Fl. control is the fluorescence of the test solution containing solvent instead of the extract and Fl. blank is the fluorescence of the test solution containing all the reagents except the enzyme.

The IC_{50} ± SD (n = 3) values were calculated in mg/mL by linear interpolation.

Renin inhibition activity

The renin inhibitory activity of the different plant extracts and fractions was determined according to the previously described method of Bhullar *et al.*[19] with some modifications. Fifty µL of renin enzyme was dissolved in the assay buffer consisting of 50-mM Tris-HCl buffer (pH 8.0) and 100-mM NaCl (assay buffer). The activated enzyme was stored at –80°C, for further analysis. Renin substrate; (Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg) was diluted with DMSO to prepare a solution of 500-µM concentration. The assay was performed using 96-well microplates. The test extracts (10 mg/mL in methanol) were prepared using 20 µL of substrate, 150 µL of assay buffer, and 10 µL of each sample. Aliskiren was used as a reference drug in concentration 0.1 mg/mL. Blank samples contained 20 µL of substrate along with 160 µL of assay buffer and 10 µL of the sample. Positive control samples were prepared with 20 µL of substrate, 150 µL of assay buffer, and 10 µL methanol. The reaction was catalyzed by adding 10 µL of renin solution to the positive control and test solutions. The reaction mixture was then incubated at 37°C for 45 min, and the fluorescence produced was measured at the excitation wavelength of 340 nm and emission wavelength of 490 nm using the FLUOstar OPTIMA plate (BMG Labtech, Offenburg, Germany). The renin inhibition was expressed as the percentage of inhibition (% I ± SD) using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Fl.control} - (\text{Fl.sample} - \text{Fl.blank})}{\text{Fl.control}} \times 100 \text{ .}$$

Results

Determination of phenolic and flavonoid contents

The phenolic and flavonoid contents of the extracts and fractions of the different organs of *Cuphea ignea* A. DC. are reported in Table 1. The results showed that the alcoholic extract of the leaves displayed the highest phenolic content calculated as gallic acid equivalent (GAE) (212.98 ± 0.13 µg GAE/mg extract), followed by the alcoholic extract of the flowers (188.25 ± 0.12 µg GAE/mg extract). In addition, the alcoholic and aqueous extracts of the leaves showed the highest flavonoidal contents (65.932 ± 0.084 µg/mg and 32.372 ± 0.44 µg/mg, respectively) calculated as quercetin equivalent.

Determination of the antioxidant activity

The antioxidant activity was performed using the DPPH and ORAC assays and the results are reported in Table 1 expressed as IC_{50} values (mean ± SD) (µg/mL). The alcoholic extracts of the leaves and the stems displayed the highest antioxidant activity with IC_{50} values of 11.15 µg/mL and 15.76 µg/mL in the DPPH assay and 10.33 µg/mL and 14.68 µg/mL in the ORAC assay, respectively, as compared with trolox.

Evaluation of *in vitro* antihypertensive activity

Angiotensin-converting enzyme inhibition assay

Overproduction of ACE catalyzes the conversion of angiotensin I to angiotensin II, leading to vasoconstriction through bradykinin inactivation and subsequently, hypertension.[20] This study investigated the ACE inhibitory potential of *Cuphea*

Table 1: Total phenolic and flavonoid contents and antioxidant activity of the alcoholic and aqueous extracts of *Cuphea ignea* A. DC.

Extract	Phenolic content (µg/mg) ± SD	Flavonoid content (µg/mg) ± SD	Antioxidant activity (DPPH) IC_{50} (µg/mL) ± SD	Antioxidant activity (ORAC) IC_{50} (µg/mL) ± SD
Leaves (Alc. ext.)	212.98 ± 0.13	65.932 ± 0.084	11.15 ± 1.26	10.33 ± 2.42
Stem (Alc. ext.)	144.31 ± 0.01	26.440 ± 0.001	15.76 ± 1.36	14.68 ± 2.01
Flowers (Alc. ext.)	188.25 ± 0.12	27.316 ± 0.129	34.55 ± 2.17	32.50 ± 1.89
Leaves (Aq. ext.)	128.57 ± 0.13	32.372 ± 0.440	23.61 ± 3.45	22.90 ± 2.27
Stem (Aq. ext.)	152.36 ± 0.14	11.356 ± 0.847	55.88 ± 1.16	52.71 ± 3.10
Ascorbic acid	–	–	1.83 ± 1.57	–
Trolox	–	–	–	27.0 ± 12.35

Alc. = alcoholic, Aq. = aqueous, ext = extract
Results displayed are the average of three independent experiments

ignea A. DC. extracts and fractions, and all the tested extracts showed significant ACE inhibitory activity as shown in Table 2 as compared with lisinopril (0.005 mg/mL). The aqueous extract of leaves showed the highest activity followed by the alcoholic extract of the flowers (IC_{50}= 0.491 mg/mL and 1.748 mg/mL, respectively). Moreover, the aqueous extract of the stems and the alcoholic extract of the leaves showed moderate activities (IC_{50}= 2.036 mg/mL and 2.151 mg/mL, respectively). Concerning the fractions, the *n*-butanol fraction of the leaves showed the highest ACE inhibition activity (IC_{50}= 0.084 mg/mL) followed by the ethyl acetate fractions of the leaves and the stems (IC_{50}= 0.215 mg/mL and 0.257 mg/mL, respectively). The methylene chloride fraction of the leaves also showed ACE inhibitory activity (IC_{50} = 2.778 mg/mL). It is worthy to mention that the *n*-butanol and ethyl acetate fractions of the leaves showed significant higher activities than their parent alcoholic extract. Also, the ethyl acetate, *n*-butanol, and petroleum ether fractions of the stems were more active against ACE than their total alcoholic extract.

Renin inhibition activity

Renin is a vital enzyme in the RAAS, where its uncontrolled overproduction leads to an increase in the level of angiotensin I in the blood, consequently leading to higher blood pressure.[19] *In vitro* renin inhibition assay was conducted to assess the renin inhibitory properties of the extracts and fractions of *Cuphea ignea* A. DC. and the results are reported in Table 2. The alcoholic extract of leaves showed the highest percentage of inhibition (94.82%) at sample concentration 10 mg/mL followed by the alcoholic extracts of the stems and flowers (88.98% and 86.65%, respectively) as compared with aliskiren, which showed 98.80% at concentration 0.1 mg/mL. Concerning the fractions, the methylene chloride fraction of the stems showed the highest renin inhibition activity (98.14%) followed by the ethyl acetate, petroleum ether, and *n*-butanol fractions of the leaves (93.09%, 92.22%, and 91.97%, respectively).

Table 2: ACE and renin inhibition activities of the different extracts of *Cuphea ignea* A. DC.

Extract	ACE inhibition IC_{50} (mg/mL) ± SD	Renin % inhibition ± SD (10 mg/mL)
Leaves (Alc. ext.)	2.151 ± 0.238	94.82 ± 2.415
Stem (Alc. ext.)	5.707 ± 0.754	88.98 ± 5.653
Flowers (Alc. ext.)	1.748 ± 0.082	86.65 ± 4.369
Leaves (Aq. ext.)	0.491 ± 0.06	70.68 ± 5.900
Stem (Aq. ext.)	2.036 ± 0.279	74.97 ± 4.618
Leaves (PE fr.)	12.26 ± 3.385	92.22 ± 1.578
Leaves (ME fr.)	2.778 ± 0.316	1.533 ± 0.503
Leaves (EA fr.)	0.215 ± 0.017	93.09 ± 2.903
Leaves (*n*-BU fr.)	0.084 ± 0.012	91.97 ± 4.297
Stem (PE fr.)	4.357 ± 0.628	81.46 ± 8.497
Stem (ME fr.)	6.831 ± 0.482	98.14 ± 0.688
Stem (EA fr.)	0.257 ± 0.038	58.76 ± 3.999
Stems (*n*-BU fr.)	3.729 ± 0.263	84.98 ± 2.723
Lisinopril	0.005 ± 0.0002	
Aliskiren[a]		98.80 ± 0.345

Alc. = alcoholic, Aq. = aqueous, ext = extract, fr. = fraction, PE = petroleum ether, ME = methylene chloride, EA = ethyl acetate, *n*-BU = butanol

Results displayed are the average of three independent experiments

[a]Standard was used at concentration 0.1 mg/mL

Correlation study

Correlation between the phenolic and the flavonoid contents as compared with the antioxidant, ACE, and renin inhibition activities was studied using Pearson's correlation coefficient (*r*) and the results are reported in Table 3. The antioxidant activity showed moderate negative correlation with the phenolic content (r = –0.507 and –0.501 for the DPPH and the ORAC assays, respectively). These results indicated the contribution of phenolics to the antioxidant activity. However, the antioxidant activity showed significant correlation with the flavonoid content (r = –0.739 and –0.734 for the DPPH and the ORAC assays, respectively). In addition, the renin inhibition activity showed significant positive correlation with the phenolic and flavonoid contents with high r values (0.777 and 0.629, respectively). Finally, the Pearson's correlation analysis did not confirm the correlation between the ACE inhibition activity and both the phenolic and the flavonoid contents.

Discussion

Inhibition of ACE and renin is considered effective strategies in the search for new natural antihypertensive agents, which could be effective, safe, and affordable. Plants rich in phenolic compounds, for example, flavonoids and phenolic acids can inhibit these two enzymes.[21] Consequently, the ACE and renin inhibitory activities where studied in this work and the results showed that alcoholic extract of the leaves alongside its fractions showed high inhibitory activities against both enzymes in addition to the aqueous extract of the leaves, which showed strong ACE inhibitory activity. These results agreed with previous reports on *Cuphea carthagenesis* (Jacq.) J.F.Macbr. in which the aerial parts showed strong ACE inhibitory potential.[14] The strong correlation between the phenolic and flavonoid contents of the alcoholic extract of the leaves showed that the phenolic constituents of the extract could be largely responsible for its enzyme inhibitory effects, especially that against renin.

Oxidative stress is a common feature of hypertension where alleviating the oxidative stress in the body is vital for management of hypertension,[22] *Cuphea ignea* A. DC. extracts showed significant antioxidant activity, which was shown using two complementary methods: DPPH radical scavenging assay and the ORAC assay. These results are in agreement with previous reports on the antioxidant activity of a coumarin compound isolated from the aqueous ethanolic extract of the aerial parts of the plant.[23] In addition, a previous study performed on *Cuphea carthagenesis* (Jacq.) J.F.Macbr. revealed that the hydroalcoholic extract of the aerial parts showed antioxidant activity with high phenolic content.[24]

Table 3: The *r* values (correlation coefficients) between phenolic and flavonoid contents related to different activities

Activity	Phenolic content	Flavonoid content
Antioxidant activity (DPPH)	–0.507	**–0.739**
Antioxidant activity (ORAC)	–0.501	–0.734
ACE inhibition activity	–0.079	–0.109
Renin inhibition activity	**0.777**	0.629

The bold-face numbers represent highest correlations

Conclusion

In this study, we investigated the antihypertensive and antioxidant activities of the different organs of *Cuphea ignea* A. DC. The results showed that the significant inhibition of ACE and renin, in addition to the strong antioxidant activities, could be the possible mechanisms for the antihypertensive activity. The different plant extracts showed high phenolic and flavonoid contents, which is correlated positively to the antioxidant activity. However, further *in vivo* studies with experimental animal models are needed to confirm the antihypertensive activity, in addition to isolation of the active phytochemical constituents responsible for the activity which could lead to the development of an effective and safe remedy of natural source for controlling hypertension.

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Conflicts of interest

There are no conflicts of interest.

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