Abstract

In the search for lead drugs for hypertension from plant origin, *Ruellia praetermissa* was analyzed for its constituents and inhibitory effects on angiotensin-converting enzyme (ACE). The various fractions showed various inhibitory effects on ACE at a concentration of 0.33 mg/ml, e.g., n-hexane extract (13.15%), ethyl acetate extract (7.15%), methanol extract (38.35%), the aqueous extract (89.65%). TLC and HPLC fingerprint analyses of these extracts showed that lupeol is one of the constituents responsible for the effect in the n-hexane extract, and flavonoids (luteolin and apigenin) might be responsible for the activity in the methanol and aqueous extracts. The aqueous extract was found to be the most active, possibly due to saponigenins. This activity suggests a cardiovascular effect of the aqueous extract of *Ruellia praetermissa*.

Keywords: *Ruellia praetermissa*; angiotensin-converting enzyme (ACE), angiotensin II.

Introduction

Each substance that exerts an inhibitory effect on ACE could play a key role in influencing blood pressure. Inhibition of angiotensin-converting enzyme is now internationally established as the therapeutic principle in the treatment of hypertension. Conceptionally, two kinds of renin-angiotensin systems exist:

- a circulatory endocrine renin-angiotensin system responsible for short term cardiorenal homeostasis
- a local renin-angiotensin system present in many tissues where they exert autocrine and paracrine effects on the local tissue functions.

The tonic control of vascular and local tissue function such as the kidney is under the control of the intrinsic tissue renin-angiotensin system. Angiotensin-converting enzyme plays an important role in the regulation of the blood pressure, acting as a dipeptidyl carboxypeptidase, the enzyme liberates angiotensin II (having vasopressor activity) from the angiotensin I which, in turn, is a product of renin. ACE is believed to be the rate-limiting step in angiotensin II formation by the renin-angiotensin system, and the kalalikrein-kinin system, to activate angiotension II and inactivate bradykinin. In addition to directly causing vasoconstriction, angiotensin II stimulates the synthesis and release of aldosterone, which also increases with blood pressure promoting sodium ion retention. ACE is identical to kinase II, an important kinin-degrading peptide; the catabolism of bradykinin to inactivate fragments also contributes to the hypertensive effects of ACE-activity. Angiotensin II also stimulates the production of superoxide anion and hydrogen peroxide in polymorphonuclear leukocytes, which inactivate the vasodilatory compounds endothelial derived vascular relaxing factor (nitric oxide-NO) and proacyclins (PGI₂) (Elbl & Wagner, 1991). If the formation of angiotensin II and the activation of vasodilatory kinins are suppressed by selective ACE-inhibitors, there will be a lowering of blood pressure.

Plant products and substances isolated from plants shown inhibitory effects on ACE (Kameda et al., 1987; Arisawa et al., 1989; Wagner et al., 1991; Wagner, 1993; Lin et al., 1994, Hansen et al., 1995; Nyman et al., 1998) In search for lead drugs for hypertension from plant origin, *Ruellia praetermissa* was investigated for ACE-inhibition.

Methodology

The principle of this screening is according to Elbl and Wagner (1991) and modified by Hansen et al. (1995). The
substrate used in the assay is chromophore- and fluorophore-
labelled tripeptide, dansyl-triglycine, which is cleaved by the
enzyme into dansylglycine and diglycine as above.

In the absence of a specific inhibitor or in presence of a
suitable environment for the ACE activity, product formation
favours an increase in blood pressure. In this situation, the
substrate, dansyltriglyceride, will be enzymatically cleaved
under the action of ACE to give dansylglycine. In the pres-
ence of a specific inhibitor, product formation will be inhib-
ited, partially or completely.

Materials and Methods

Plant materials

The plant material was collected in Elemighong-Cameroon
in three different habitats and in different seasons of the year,
August 1996, December 1997 and March 1998. The speci-
men was identified and authenticated by Mr. Kofany of the
Cameroon National Herbarium where a voucher specimen
number 43596 was deposited.

Extraction and sample preparation

The sun-dried leaves were pulverized and 150 g Soxhlet
extracted for 6 h in each case progressively with 1 liter of n-
hexane, dichloromethane, EtOAc, and MeOH, and extracted
fractions were recovered by rotary evaporation. The chaffs
were boiled in water at 85 °C for 4 h and the extracts were
recovered by lyophilization. Small test amounts were chro-
matographed in SEP-Pak® cartridges with methanol as the
eluent to eliminate chlorophyll. The plant extracts were pre-
pared by dissolving 1 mg of chlorophyll free extract in 1 ml
of methanol. The extracts were filtered using a 0.45 μm,
membrane filter (Altech, Germany) after centrifugation.

TLC and HPLC fingerprint analysis

TLC was done using Silica gel 60F254 prep-prepared plates
(Merck, Darmstadt, Germany) and ethyl acetate-formic acid-
glacial acetic acid-water (100:11:11:26) and chloroform-
methanol-water (64:50:10) as the mobile phases. Plates
were examined in UV254 and UV366 nm before and/or after
spraying with NP/PEG or AS spraying agents.

The HPLC was carried out with an HP 1090 A liquid
chromatograph and HP 1040 A photodiode array with a HP
9000/300 personal computer on a LiChrospher® 100 RP 18
(5 μm) column 125 x 4 mm. The mobile phase used for the
separation was water (solvent A) and acetonitrile (solvent B)
each containing 0.05% H3PO4 at a flow rate of 1 ml/min.
Elution was started with 10% B followed by a linear gradi-
ent of 10–30% MeCN during 30 min with detection at 210,
254 280 and 366 nm.

ACE test methods

The plant material

The chlorophyll free n-hexane, dichloromethane, ethylac-
etate, methanol and the aqueous extracts of Ruellia praeter-
missa were evaporated to dryness and the test sample was
made by dissolving 1 mg of dry extract in 20% methanol
(test solution 1) and 1 mg in HEPES assay buffer (test solu-
tion 2).

Reference substances

Reference substance 1 = 20% methanol.
Reference substance 2 = 80% HEPES.

Chemicals

Dansyltriglycine was purchased from Hoechst AG
(Frankfurt, Germany); dansylglycine and dansyl-t-glutamic
acid, from Sigma Chemical Co (Taufkirchen, Germany);
angetinsin-converting enzyme from rabbit lung was pur-
chased from Sigma Chemical Co (Taufkirchen, Germany).

Buffers

- Assay buffer: HEPES-NaOH, 50 mM, pH 8.0, containing
300 mM NaCl (11.92 g HEPES and 17.52 g NaCl were
dissolved in 900 ml distilled water and adjusted to pH 8.0
with NaOH and distilled H2O to 1 L).
- Elution buffer for HPLC: NaH2PO4-NaOH, 10 mM,
pH 7.0 (1.56 g NaH2PO4.2H2O were dissolved in 900 ml
distilled water and was adjusted to 7.0 with NaOH and
distilled H2O to 1 L). Before use, all the buffers were fil-
tered through millipore sterile-filter HA filter unit with a pore
of 0.45 μm.

Enzymes

One unit of angiotensin-converting enzyme with the specific
activity of 3 units/mg protein was dissolved in 2500 μl assay
buffer.
Stock solution: 1.66 mg dansyltriglycine was dissolved in 500 μl HEPES assay buffer.

Working solution: The stock solution was diluted to 166 μg/ml with HEPES assay buffer (45 μl stock solution plus 855 μl HEPES assay buffer). To this solution, 600 μl internal standard solution (13.43 mg dansyl-l-glutamine dissolved in 100 ml HEPES assay buffer) was added.

Biological assay: The enzyme assay was performed in the following manner. Using microtitre plates, test (inhibitor) solution or assay buffer (control incubation) were added to 25 μl of enzyme solution. The microtitre plate was kept for 5 min in a water bath at 37°C, and the reaction was started by adding 25 μl substrate (0.236 mM) and internal standard solution. After an incubation at 37°C, the reaction was stopped by adding 50 μl 0.1 N Na₂EDTA. The incubation was time dependent on the activity of the applied enzyme batch, which was determined for each enzyme batch. Following termination of the reaction, solutions were transferred to HPLC tubes (CS Chromatographic Service, Eschborn, Germany), and the product and the unreacted substrate were separated and quantified by reversed phase HPLC with detection at 250 nm. Peaks were identified by co-chromatography with authentic references substances and by on-line spectra.

Test evaluation with the method of internal standardization

The absolute amount of dansylglycine in the reaction and the control reaction was evaluated according to the following formula:

\[
\text{Amount of dansylglycine} = \frac{\text{Area of peak DG} \times \text{Amount of IS}}{\text{CF} \times \text{Area of peak IS}}
\]

Where:

- DG = dansylglycine
- IS = internal standard
- CF = calibration factor of dansylglycine relative to the internal standard with CF = 1.121.

The decrease in the concentration of dansylglycine in the test reaction compared to the control reaction, was expressed as percent inhibition, calculated from the equation:

\[
\text{Inhibition} (\%) = 100 - \frac{\text{Dansylglycine}_T}{\text{Dansylglycine}_C} \times 100
\]

Where:

- T = test reaction
- C = control

Results and Discussion

The results are presented in Figure 1.

Strong inhibitory activity was observed for the aqueous extract (89.65%) and other extracts showed relatively weak inhibitory activity: n-hexane extract (13.15%), EtOAc extract (7.15%), MeOH extract (38.35%) and the sapogenin isolated from the plant (50.54%). This observation in theory implies that aqueous extract of Ruellia praetermissa can constitute a good anti-hypertensive agent. The n-hexane extract contains amongst other chemical constituents, lupeol, having a hypotensive effect with one mechanism of action being interaction with ACE (Harborne & Baxter, 1983). The EtOAc, MeOH and the aqueous extracts have apigenin as one of the flavone aglycones, which shows an antihypertensive effect by both affecting ACE and calcium channel blocking activity. In the aqueous extracts is a sapogenin (kawan), a diterpene isolated from these extract which in a pure form showed a 50.54% inhibition of ACE. The combination of the effects of this compound and apigenin in the aqueous extract might be responsible for its strong inhibitory effect. On the other hand, the presence of a strong ACE inhibition might not necessary imply that the species is a potential anti-hypertensive drug (Wagner et al., 1991), since flavonoids may show an in vitro activity due to the generation of chelation complexes within the active center of ACE (Wagner, 1993; Lin et al., 1994; Kameda et al., 1987). The MeOH and aqueous extracts of Ruellia praetermissa are rich in flavonoids such as luteolin-7-glucoside, apigenin-8-glucoside, luteolin, apigenin and apigenin-7β-D-glucuronic acid. Some of these flavonoids might be responsible for the positive results obtained for these extracts, due to their capacity of generating in vitro chelation complexes. Flavonoids ability to form chelates with the active center of ACE depend on the substitution pattern of the hydroxy groups suggesting that these molecules might produce inhibition effects by complexation with the zinc atom of the enzyme (Wagner et al., 1991).

The values above for the percentage of activity of Ruellia praetermissa extracts on ACE inhibition are based on crude extracts values. ACE inhibition in the range of 35–50% should be considered negative in crude extracts (Hansen et al., 1995) but a negative result does not necessary mean that this plant species is inactive as an anti-hypertensive plant drug (Hansel et al., 1995; Nyman et al., 1988) as compounds influencing other hypotensive mechanisms such as beta-
receptor blockers and calcium antagonists could be present. From these observations, we can conclude that the aqueous extract of *Ruellia praetermissa* inhibits ACE. Investigation of the aqueous extract for calcium channel blocking activity is in progress to confirm if the inhibition of ACE implies anti-hypertensive activity.

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**References**


