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# Screening of some Yemeni medicinal plants for inhibitory activity against peptidases

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Extracts of different polarities (dichloromethane, methanol, and aqueous extracts) from 5 Yemeni medicinal plants (*Aspilia helianthoides*, leaves; *Ceropegia rupicola*, whole plant; *Kniphofia sumarae*, whole plant; *Pavetta longiflora*, leaves; and *Plectranthus* cf *barbatus*, leaves) were screened for their inhibitory effects against angiotensin converting enzyme (ACE), neutral endopeptidase (NEP), and aminopeptidase N (APN) activities. Four extracts (methanol extracts of *Ceropegia rupicola*, *Kniphofia sumarae*, and *Plectranthus* cf *barbatus*, and the aqueous extract of *Pavetta longiflora*) were found able to inhibit the enzymatic activity of NEP. Significant reduction in the activity of NEP ($p < 0.01$) was observed at a concentration of 50 μg/ml, and above of all tested extracts. The most active extract was the methanolic extract of *Ceropegia rupicola* with $IC_{50}$ of 111 μg/ml. Only the methanolic extract of *Aspilia helianthoides* was found to exhibit inhibitory effect against the ACE activity with $IC_{50} = 133$ μg/ml. None of the tested plant extracts was found active against the aminopeptidase N activity.

## 1. Introduction

Herbal medicine, as an important part of Yemeni traditional medicine, has managed to survive till the present almost intact thanks to the rich and diverse endemic flora in Yemen and to the inheritance of a very long experience in phytotherapy used by indigenous people in traditional healing of several diseases (Fleurentin and Pelt 1982, 1983). The purpose of our work was to test some Yemeni medicinal plants for their inhibitory activity against peptidases such as neutral endopeptidase (NEP), angiotensin-converting enzyme (ACE) and aminopeptidase N (APN). Such a screening provides a quick estimation of the bioactivity of the tested plants against the three peptidases, which are involved in a number of physiological and pathological processes in human beings, and thus indicating a possible role of medicinal plants as a useful source to be further explored for the development of therapeutic agents.

Neutral endopeptidase (known as neprilysin, enkephalinase, CALLA or CD10), angiotensin-converting enzyme, and aminopeptidase N are members of zinc metallopeptidases, acting as ectoenzymes on the outer surface of different cells and are involved in the metabolism of several regulatory peptides of human nervous, cardiovascular, inflammatory and immune systems. ACE forms the hypertensive peptide angiotensin II from inactive precursor angiotensin I. APN and NEP together inactivate the endogenous opoid peptides enkephalins, however NEP is also the major enzymatic pathway for degradation of natriuretic peptides, which counteract the renin-angiotensin-aldosterone system and endothelin.

Despite the documented clinical efficacy of ACE inhibitors for the treatment of hypertension and congestive heart failure, a substantial number of hypertensive patients are not adequately controlled with ACE inhibitors monotherapy. Clinical experiences with NEP inhibitors were not successful either. On the other hand a new class of dual metallopeptidase inhibitors of ACE and NEP, the so called vasopeptidase inhibitors, are found more effective in the treatment of cardiovascular diseases. The synergistic effect of combined NEP and ACE inhibition is a consequence of inhibiting the renin-angiotensin-aldosterone system and potentiating the natriuretic peptide and kinin system. So the vasopeptidase inhibitors reduce vasoconstriction and enhance vasodilation, improve sodium/water balance, and, in turn decrease peripheral vascular resistance as well as blood pressure, and improve local blood flow (Floras 2002; Veelken and Schmieder 2002; Worthely et al. 2004). APN inhibitors as well as dual APN/NEP inhibitors are other targets of scientific research to develop anticancer and anti-inflammatory drugs as well as a new class of analgesics devoid of morphine side effects respectively. In addition, the dual APN/NEP inhibitors demonstrate antidepressant-like properties through enkephalin-related activation of delta-opoid receptors (Le Guen et al. 2003; Bauvois and Dauzonne 2006; Noble and Roques 2007). Recent work (Salazar-Lindo et al. 2000; Wang et al. 2005; Farthing 2006) indicated enkephalinase inhibitors (NEP inhibitors), which potentiate the effects of endogenous enkephalin activity, have antisecretory and antidiarrheal actions and can be used as a safe and effective treatment for acute diarrhea in adults and children. Racecadotril (Thiorphan)

**Table: Influence of different extracts of some Yemeni medicinal plants on NEP activity**

| Plant sample | NEP activity (% of control) | | | | | | $IC_{50}$ NEP (µg/ml) |
|---|---|---|---|---|---|---|---|
| | 1 µg/ml | 10 µg/ml | 25 µg/ml | 50 µg/ml | 100 µg/ml | 200 µg/ml | |
| *Ceropegia rupicola* Methanolic extract | 89 ± 4 | 83 ± 5* | 69 ± 4** | 51 ± 7 ** | 54 ± 3** | 29 ± 14** | 111 |
| *Kniphofia sumarae* Methanolic extract | 98 ± 4 | 88 ± 4* | 76 ± 4** | 62 ± 9** | 62 ± 3** | 37 ± 14** | 141 |
| *Pavetta longiflora* Aqueous extract | 97 ± 5 | 87 ± 4 | 77 ± 3 | 65 ± 3** | 64 ± 7** | 37 ± 9** | 144 |
| *Plectranthus cf. barbatus* Methanolic extract | 93 ± 4 | 97 ± 3 | 86 ± 5* | 71 ± 14** | 61 ± 3** | 33 ± 13** | 139 |

NEP activity of control: 100 ± 4%. * Significant difference to control at $P < 0.05$, ** Significant difference to control at $P < 0.01$

is an example of an NEP inhibitor that is used effectively for the treatment of acute watery diarrhea in adults and children.

## 2. Investigations, results and discussion

Out of 15 extracts tested for their inhibitory effects against ACE, NEP and APN, 4 extracts (methanol extracts of *Ceropegia rupicola*, *Kniphofia sumarae*, and *Plectranthus* cf. *barbatus*, and the aqueous extract of *Pavetta longiflora*) were found able to inhibit the enzymatic activity of NEP (Table). Significant reduction in the activity of NEP ($p < 0.01$) was observed at a concentration of 50 µg/ml, and above of all tested extracts. The most active extract was the methanolic extract of *Ceropegia rupicola* with $IC_{50}$ of 111 µg/ml. It reduced the enzymatic activity of NEP to ca. 53% by a concentration of 50–100 µg/ml (47% inhibition of NEP activity), and to 29% by a concentration of 200 µg/ml (71% inhibition of NEP activity). Other tested extracts however showed similar significant reduction ($p < 0.01$) in the NEP activity at the concentration of 50 µg/ml, and above (Fig. 1).

Although the tested plants are used for purposes such as skin diseases and as anti-malarial and homeostatic agents (Fleurentin and Pelt 1982), related species are used in other countries as analgesics, anti-diarrheal, and antihypertensive agents and for the treatment of congestive heart failure (Sukumar et al. 1995; Amos et al. 2003; Wube et al. 2005; Lukhoba et al. 2006). Due to the importance of NEP inhibitors in the management of hypertension, diarrhea and pain, the presence of NEP inhibitors in the tested plant extracts that is proven in our study suggests that these plants could be useful sources for further bioassay-guided investigation to isolate and characterize active constituents responsible for the inhibitory effect, which might be useful as new structures for the development of new antihypertensive, anti-diarrhoeal, and analgesics agents.

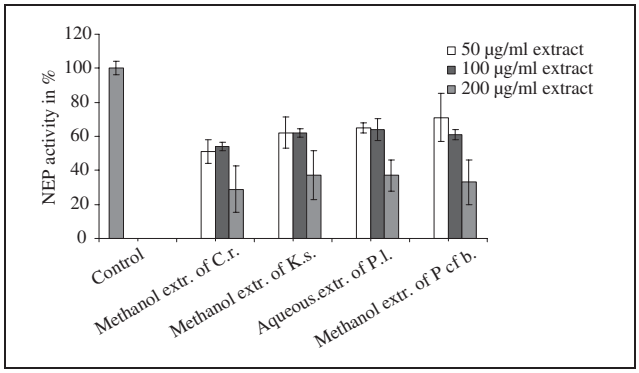


Fig. 1: Influence of methanolic extracts of *Ceropegia rupicola* (C.r.), *Kniphofia sumarae* (K.s.), *Plectranthus* cf. *barbatus* (P. cf. b.), and aqueous extract of *Pavetta longiflora* (P.l.) on the NEP activity

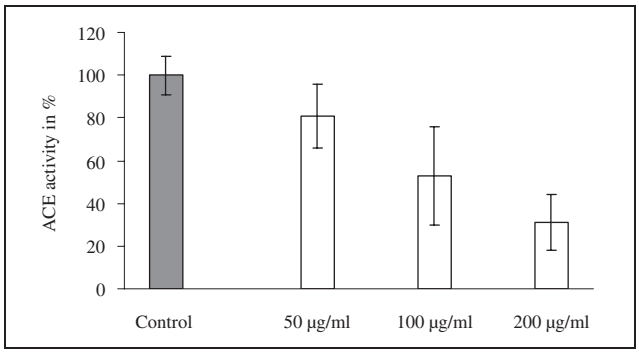


Fig. 2: Influence of different concentrations of methanolic extracts of *Aspilia helianthoides* on ACE activity

Only the methanolic extract of *Aspilia helianthoides* was found to exhibit inhibitory effect against ACE activity (Fig. 2). It shows a significant reduction ($P < 0.02$) of the ACE activity to 31% compared to the control in a concentration of 200 µg/ml (69% inhibition of ACE activity). Considering the importance of ACE inhibitors in the treatment of hypertension, the plant material – although its inhibition on ACE with $IC_{50} = 133$ µg/ml seems to be moderate – deserves further bio-guided isolation of the inhibitory active constituents that may play a role for the development of antihypertensive agents.

None of the tested plant extracts was found active against aminopeptidase N activity.

## 3. Experimental

### *3.1. Materials*

Plants used in this study were collected in Taiz province-Yemen. The authentication of the plants was made under the supervision of Dr. A. W. Al-Khulaidi at the Agriculture Research Center in Dhamar-Yemen. The plant materials were shade dried at ambient temperature and then ground in a grinder.

Suc-L-Ala-L-Ala-Phe-7-amino-3-methyl-coumarin (SAAP-AMC), Hip-L-His-L-Leu, phosphoramidon, Aminopeptidase (leucine aminopeptidase, type IV-S from porcine kidney microsomes), lisinopril, and L-Leucine-*p*-nitroanilide were purchased from Sigma. Phthaldialdehyde was obtained from Merck. Source of NEP was a boar sperm preparation provided by Dr. Siems as well as an ACE preparation from rat kidney and lung (Research Institute for Molecular Pharmacology, Berlin, Germany).

### *3.2. Preparation of extracts*

A series of subsamples (50 g each) of each plant were extracted with a solvent (dichloromethane, methanol and distilled water) under shaking, for 3 h. at ambient laboratory temperature, and then in ultrasonic bath for 20 min. The extraction was performed 3 times. The collected filtered extracts were then concentrated to dryness. A series of concentrations (1, 10, 25, 50, 100, and 200 µg/ml) of the dichloromethane and methanol extracts dissolved in dimethylsulfoxide (DMSO), and of the aqueous extract dissolved in distilled water were prepared for the test.

### *3.3. Determination of enzyme activity*

Enzyme activities were determined according to Bormann and Melzig (2000).

For the determination of ACE activity, 20 µl of Hip-L-His-L-Leu solution (4 mM in water) were added to 250 µl of phosphate-buffer (83 mM $K_2HPO_4$ + 326 mM NaCl, pH 8.3), 10 µl of extract solution and 20 µl of ACE. The mixture was incubated for 30 min at 37 °C, and stopped with 0.4 M NaOH (1000 µl). Methanolic o-phthaladehyde solution (2%, 100 µl) was then added to produce the fluorescence His-Leu-*o*-phthalaldehyde complex. Under the exclusion of light the mixture was incubated for 10 min, and the reaction was terminated by adding 300 µl of 2 M HCl. This mixture was incubated for a further 30 min in dark place and then the fluorescence was measured at 365 $nm_{exit}$/500 $nm_{emiss.}$ The remaining activity of the ACE (as % of the control) was calculated in comparison to the control without inhibitor, considering the influence of the solvent, and test extract.

For the determination of NEP activity a two steps assay was used. 50 µl of lisinopril (8 µM), 50 µl of SAAP-AMC (400 µM), 390 µl of HEPES-buffer (50 mM + 154 mM NaCl, pH 7.4) and 10 µl extract or without extract were mixed. The first enzymatic reaction was started by adding 150 µl of diluted (1 : 800) boar sperm preparation to the mixture and incubated for 60 min at 37 °C. The reaction was stopped by the addition of 50 µl of phosphoramidon solution (50 µM). The second enzymatic reaction started after an addition of 20 µl of APN-solution (1 : 235) to the mixture and incubation for 60 min at 56 °C. The reaction was terminated by adding 800 µl of acetone. The fluorescence of the released AMC was measured at 367 $nm_{exit}$/440 $nm_{emiss}$. The remaining activity of NEP (as % of the control) was calculated in comparison to the control without inhibitor, considering the influence of the solvent, and test extract.

To determine the activity of APN, 250 µl of L-leucine-*p*-nitroanilide solution (2 mM in HEPES-buffer) was added to 190 µl of HEPES-buffer (50 mM + 154 mM NaCl, pH 7.4) with 10 µl test extract or without test extract. The reaction was started by adding 50 µl of APN solution (1 : 5000 in HEPES-buffer) and incubating for 60 min at 37 °C. The addition of 800 µl of acetone stopped the reaction. The samples were measured spectrophotometrically at 405 nm to determine the formed *p*-nitroaniline. The remaining activity of APN (as % of the control) was calculated in comparison to the control without inhibitor, considering the influence of the solvent, and test extract.

### *3.4. Statistics*

The assay was performed three times with triplicate parallel samples. The remaining activity of the enzyme was calculated as percentage to the control without inhibitor. All values were expressed as mean ± standard deviations. Wilcoxon's U-test was used to test the significance. $IC_{50}$ values were obtained from dose-effect curves by linear regression.

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