Phytomedicine Vol. 2 (4), pp. 319–325, 1996
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Isolation of an Angiotensin Converting Enzyme (ACE) inhibitor from *Olea europaea* and *Olea lancea*

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Summary

The aqueous extract of the leaves of *Olea europaea* and *Olea lancea* both inhibited Angiotensin Converting Enzyme (ACE) *in vitro*. A bioassay-directed fractionation resulted in the isolation of a strong ACE-inhibitor namely the secoiridoid 2-(3,4-dihydroxyphenyl)ethyl 4-formyl-3-(2-oxoethyl)-4 *E*-hexenoate (oleacein) (IC_{50} = 26 μM). Five secoiridoid glycosides (oleuropein, ligstroside, excelcioside, oleoside 11-methyl ester, oleoside) isolated from Oleaceous plants showed no significant ACE-inhibition whereas, after enzymatic hydrolysis, the ACE-inhibition at 0.33 mg/ml was between 64% to 95%. Secoiridoids have not been described previously in the literature as inhibitors of ACE. Oleacein showed a low toxicity in the brine shrimp *(Artemia salina)* lethality test (LC_{50} (24 h) = 969 ppm).

Key words: *Olea europaea, Olea lancea*, olive leaves, plant, angiotensin converting enzyme, ACE, anti-hypertensive effect, hypotensive agents, secoiridoid aglycone, terpenoids, oleacein, brine shrimp, *Artemia salina*, toxicity.

Introduction

In the treatment of hypertension and cardiac failure, inhibition of Angiotensin Converting Enzyme (ACE) is established as one modern therapeutic target. The principal pharmacological actions of ACE inhibitors are vasodilation, increased sodium excretion, diuresis, and lowering of the blood pressure (Abrams et al., 1984). The influence of the renin-angiotensin system (RAS) on blood pressure has been well studied (Fig. 1). ACE acts within the RAS and the kallikrein-kinin system to activate angiotensin I into angiotensin II and to inactivate bradykinin. Several lines of evidence suggest that the RAS consists of two compartments, one in circulation and the other in local tissues. It is believed that the principal function of the circulating endocrine RAS is to provide short-term cardiorenal homeostasis. The tonic control of vascular resistance and local tissue function seems to be influenced by the intrinsic tissue RAS through more chronic processes such as secondary structural changes (Chai and Johnson, 1995; Dzau, 1988; Lee et al., 1993).

Olea lancea lam. is used by herbalists as a remedy (often combined with other plant species) to treat constipation, diabetes, poliomyelitis, pulmonary tuberculosis, mesenteric inflammation, skin rashes, as an aphrodisiac, an emmenagogue and an abortifacient, against fever, to relieve asthma symptoms in children, to improve memory, and to treat hypertension (Fakim, 1990; Lavergne, 1990; Sussman, 1980). To date no phytochemical work has been published on *O. lancea*.

The hypotensive action of olive leaf (*Olea europaea* L.) has been known for some time. Review's on the experimental and clinical studies of the hypotensive action was published as far back as in 1954 by Esdorn and in 1959 by Kosak and Stern. Investigations aimed at elucidating the active principles of the olive leaf has been conducted for decades. The bitter glycoside oleuropein has a hypotensive, coronary

dilatating and antiarrhythmic action (Petkov and Manolov, 1972). The vasodilatory effect of a decoction of olive leaves on isolated rat aorta preparations made likely that oleuropein is not solely responsible for the hypotensive effect. The presence of at least one more compound which is either a vasodilator itself or else potentiates the relaxant effect of oleuropein was indicated (Zarzuelo et al., 1991). Recently a bioassay directed fractionation revealed that β-(3,4-dihydroxyphenyl)ethanol is a potential calcium antagonist (Rauwald et al., 1994).

In our screening programme of medicinal plants for their Angiotensin Converting Enzyme (ACE) inhibitory activity an aqueous extract of both *O. europaea* and of *O. lancea* was observed to inhibit ACE significantly *in vitro*. In the present study we set out to elucidate the compound(s) mainly responsible for this activity.

Materials and Methods

Plant material

Leaves of *Olea europaea* L. (Oleaceae) were collected in May 1994 in Mallorca, Spain. The plant material was air-dried at 40 °C, powdered and extracted in the end of July 1994. Voucher specimens of the material is deposited at the Department of Pharmacognosy.

Olea lancea Lam. (Oleaceae) is indigenous to Réunion Island, Mauritius, and Rodrigues. Synonym: *Steganthus lancea* (Lam.) Knobl.; Vernacular names: *Bois d'olive blanc* (Réunion), *Bois de cerf* (Mauritius) and *Bois Malaya* (Rodrigues) (Lavergne, 1990). The leaves of *O. lancea* were collected at Colorado (Réunion) close to St. Denis in a hot and humid wet forest at an altitude of approximately 600 m in November 1994. The plant material was air-dried immediately after the collection. A voucher specimen is deposited in Herbarium C (A. & H. Adsersen 5500). The plant material was powdered and extracted in January 1995 for isolation of the active principle.

Instruments and general methods

Detection of column chromatographic fractions or the isolated compound was done on pre-coated silica gel plates, Kieselgel 60 F_{254} (Merck), using two different solvent systems as eluents: 1) EtOAc/MeOH/H_2O, 100:13.5:10 using $FeCl_3$ (and $UV_{254\,nm}$ light) to visualize compound *1* (R_f = 0.8 bluegrey); 2) CH_2Cl_2/MeOH, 9:1 using $FeCl_3$ to visualize compound *1* (R_f = 0.55).

The purity of the compound was measured by HPLC using a Shimadzu (Kyoto, Japan) instrument equipped for UV- and photodiode array detection. Instrumentation: C-R 4 A Chromatopac system with an SCL-6 A system controller, LC-6 A pumps, a CTO-6 A column oven (40 °C), an SPA-6 A UV detector (280 nm) and an UV/VIS Photodiode Array detector model SPD-M 6 A. Solvent system: Column: LiChrospher® 100 RP-18 (5 µm) in LiChroCART® 125-4; Guard column: LiChrospher® 100 RP-18 (5 µm) in LiChroCART® 4-4; solvent A: H_2O/HCOOH, 95:5; solvent B: MeOH; elution condition: isocratic 20% B, 3 min.; linear gradient from 20% B to 65% B, 7 min.; isocratic 65% B for 3 min.; flowrate 1.5 ml/min.; retention time (t_R) = 9.0 min for compound *1* (oleacein).

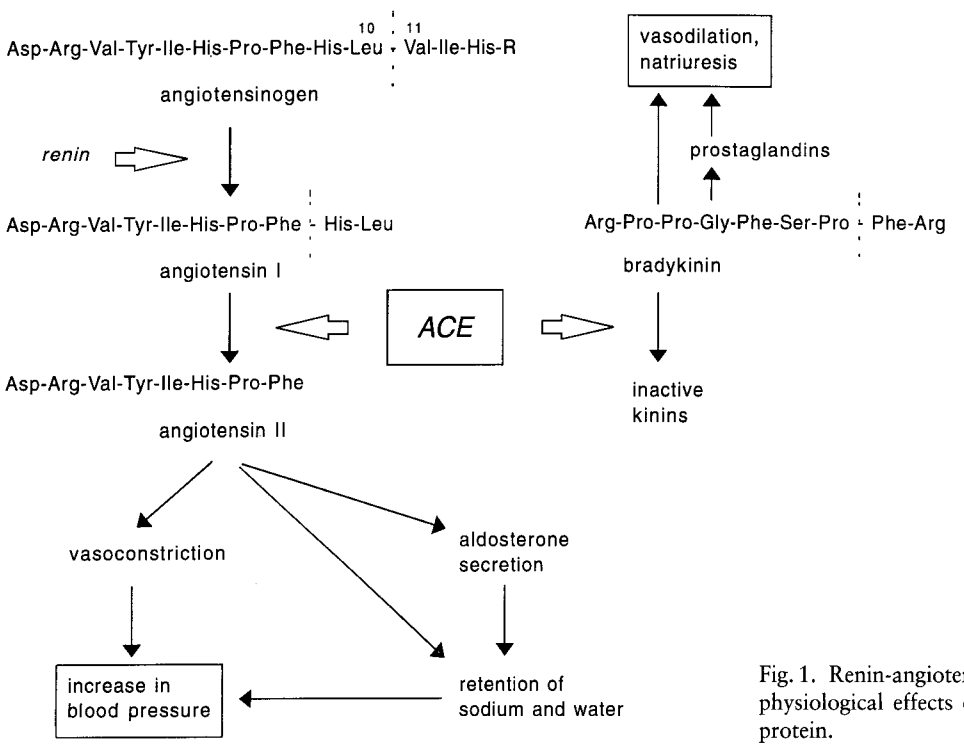


Fig. 1. Renin-angiotensin system (RAS) – release and physiological effects of angiotensin II. R, remainder of protein.

NMR spectra were recorded using a Bruker AMX 400 (2D experiments) and a Bruker AX 200 F instrument with TMS as the internal standard.

ACE-Assay

In vitro ACE inhibitory activity was measured as described by Elbl and Wagner (1991) and later modified by Hansen et al. (1995) using angiotensin converting enzyme from rabbit lung (EC 3.4.15.1 – purchased from Sigma) and dansyltriglycine as substrate. K_m = 1.22 ± 0.095 mM; IC_{50} value of 12 ± 2.6 nM for captopril.

Isolation of active substance (oleacein)

Olea europaea

The compound was isolated by a bioassay-directed fractionation each time proceeding with the most active fraction. The dried and powdered leaves (593 g) were extracted twice with 5.9 l of water on an ultra-sonic bath for 1 hr (93% ACE inhibition at 333 µg/ml of residue) and by partitioning the activity was transferred to ethylacetate (4 x 12 litres ethylacetate). The ethylacetate layer was concentrated under reduced pressure at 45 °C to give 15.45 g of a gum (97% ACE inhibition at 333 µg/ml, 58% inhibition at 33.3 µg/ml). The residue was fractionated by vacuum chromatography over silica gel (ICN Biomedical, ICN Silica TSC, 60 A, No. 04526) using accelerating gradients from dichloromethane to ethylacetate to methanol. The fraction eluted with CH_2Cl_2/EtOAc, 50:50 and containing the compounds with R_f approximately 0.8 (large yellow/brownish spot in visible light) in TLC system 1 was concentrated. The 3.94 g of residue (83% ACE inhibition at 33.3 µg/ml) was further fractionated over silica gel (Merck Kieselgel 60 reinst 70–230 mesh art. 7734 + 10% water) using CH_2Cl_2/MeOH, 95:2 as an eluent. The fractions containing the bluegrey ($FeCl_3$) compound with R_f 0.55 in TLC system 2 were combined and concentrated to obtain 2.22 g of material (66% ACE inhibition at 33.3 µg/ml). A portion (750 mg) was partitioned between diethyl ether and 5% $NaHCO_3$ (120 ml), and after washing with H_2O, 267 mg of crude oleacein (*1*, 2-(3,4-dihydroxyphenyl)ethyl 4-formyl-3-(2-oxoethyl)-4 *E*-hexenoate) was obtained from the ether layer giving an intense bluegrey spot (R_f of 0.55) in TLC system 2. Final purification was achieved by preparative TLC in system 2 using 0.5 mm precoated plates (Merck Kieselgel 60, 0.5 mm). The NMR-data of oleacein were found to be identical to those reported by Montedoro et al. (1993) and Scalzo and Scarpati (1993).

O. lancea

The dried and powdered leaves (10 g) were extracted three times with 100 ml of water on an ultra-sonic bath for 1 hour (79% ACE-inhibition at 333 µg/ml when concentrated). The water extract (300 ml) was extracted with hexane (3 x 300ml) and the hexane layer was discarded (8% ACE-inhibition at 333 µg/ml when concentrated). The defatted water layer (100% ACE-inhibition at 333 µg/ml of residue) was extracted with diethyl ether (3 x 300 ml) and the organic extract was concentrated (86% inhibition at 33.3 µg/ml). The residue (199 mg) was partitioned between diethyl ether and 5% $NaHCO_3$ (pH = 7.5) (20 ml). The organic phase was washed with brine solution and water and taken to dryness (56 mg). Final purification of oleacein *(1)* was achieved by preparative TLC (Merck Kieselgel 60, 0.5 mm pre-coated) using hexane/dichloromethane/methanol, 33:60:7 as an eluent (18 mg). In this TLC system oleacein has a R_f, value of 0.29 turning brownish green when treated with anisaldehyde/H_2SO_4 and heated at 100 °C for 10 minutes. NMR-data of the isolated compound were found to be identical to those of oleacein *(1)* isolated from *O. europaea*.

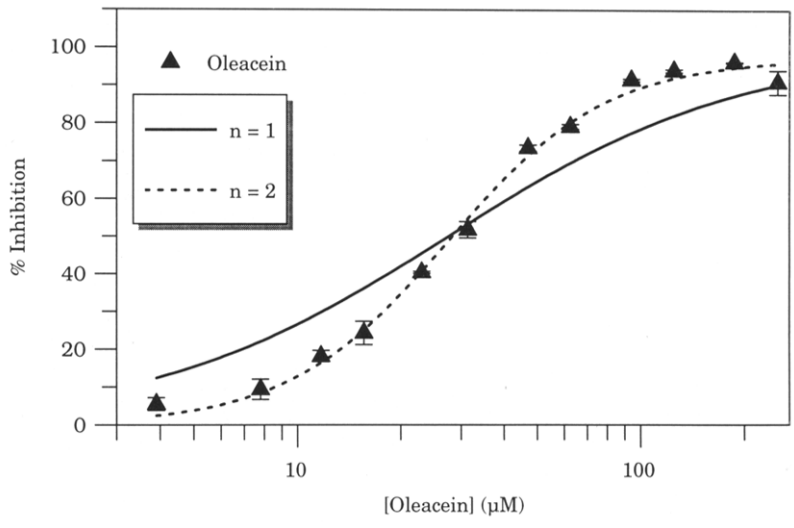


Fig. 2. Determination of the IC_{50} value of oleacein *(1)* by non-linear iterative curve-fitting of the data to the equation

$$\%\ inhibition = \frac{100\% \times [inhibitor]^n}{[IC_{50}]^n + [inhibitor]^n}$$

using the program GraFit 3.0 (Erithacus Software, Stainess, UK). IC_{50} = 26 ± 1.8 µM, n = 1.83 ± 0.12. The IC_{50} value is reported as the mean ± standard deviation of 3 separate experiments (S.D.). Vertical bars represent standard deviation. Solid line represent fit when n = 1, dotted line when n = 2.

Determination of the IC_{50} values

The IC_{50} value is reported as the means ± standard deviation (S.D.) of 3 experiments. The IC_{50} value was determined according to Fig. 2 by non-linear iterative curve-fitting of the data to the equation

$$\% \; inhibition = \frac{100\% \times [inhibitor]^n}{[IC_{50}]^n + [inhibitor]^n}$$

using the program GraFit 3.0 (Erithacus Software, Stainess, UK) (Ebert et al. 1994).

Synthesis of β-(3,4-dihydroxyphenyl)ethanol

Approximately 250 mg of verbascoside isolated from *Fraxinus excelsior* (Damtoft et al., 1992) was boiled in 1 N H_2SO_4 (10 ml) for 2 hrs. The reaction mixture was neutralized with NaOH to pH 7.5 and extracted with EtOAc (4 x 15 ml). The dried (Na_2SO_4) EtOAc layer was evaporated in vacuo. The residue was purified by preparative TLC (kieselgel 60, EtOAc/dioxane/H_2O, 30:10:0.3, detection: $FeCl_3$, grey/green middle-compound of three (R_f value 0.76) to give 7.5 mg of a syrupy substance, which was identified as β-(3,4-dihydroxyphenyl)ethanol by ^{1}H-NMR.

In situ enzymatic cleavage of secoiridoids and measurement of ACE-inhibition

The secoiridoids tested had all been isolated from plants from the Oleaceae (Damtoft et al., 1992, 1993 and 1995), except for morroniside which was isolated from young shoots of *Sambucus nigra* (Jensen and Nielsen, 1974).

0.8 mg β-glucosidase (EC 3.2.1.21) from almonds (Sigma) was dissolved in 900 µl H_2O and 2 mg secoiridoid dissolved in 100 µl MeOH was added (approximately 5 mM secoiridoid). After incubation for 2 hrs at 37 °C the completion of the enzymatic cleavage was measured with TES-TAPE®, Eli Lilly & Co., measuring the concentration of released glucose. A colour reaction of TES-TAPE® corresponding to approximately 1/10% glucose in the reaction mixture (approximately 5 mM) was taken as proof for complete cleavage. After completion of enzymatic reaction the mixtures were lyophilized and redissolved for ACE-inhibitor testing at the same theoretical molar concentration as the respective secoiridoids.

The secoiridoids were all tested for ACE-inhibitory activity at a concentration of 0.33 mg/ml.

Brine Shrimp (Artemia salina) Lethality Test

The test was carried out as described previously by Sam (1993) using newly hatched brine shrimp larvae (nauplii) collected 48 hrs after sowing. 96-well microplates were used as described by (Solis et al., 1993). Approximately 25 mauplii suspended in 100 µl artificial seawater was transferred to each well and 100 µl of a serial dilution of oleacein *(1)* dissolved in artificial sea-water/methanol, 92.5:7.5 was added. 6 wells are used for each concentration and 6 control wells with artificial seawater/methanol, 92.5:7.5 were included in the test. After incubation at 22–24 °C for 6 hrs (acute LC_{50}) and 24 hrs (chronic LC_{50}) the numbers of dead (non-motile) and total numbers of nauplii at each concentration were counted under a stereomicroscope (Wild, M. & Z. Heerbrugg, Switzerland). Potassium dichromate was used as a positive control. Treatment of quantitative data was carried out by the Reed-Muench method (Ipsen and Feigl, 1970). This procedure assumes that an animal that survived at a given concentration would also have survived at any lower concentration and that an animal that died at a certain concentration would also have died at any other higher concentration. The concentration that kill 50% of the animals (LC_{50} value) was obtained by intersection (Fig. 3). Standard error (SE) was estimated by

$$SE\ LC_{50} = \sqrt{(0.79\ h/R/n)}$$

where h = average of the interval between dosages (log dose), R = interquartile range (log LC_{75} – log LC_{25}), n = number of animals (or the average – n_{av}).

The 95% confidence limits of the LC_{50} was derived from the relationship

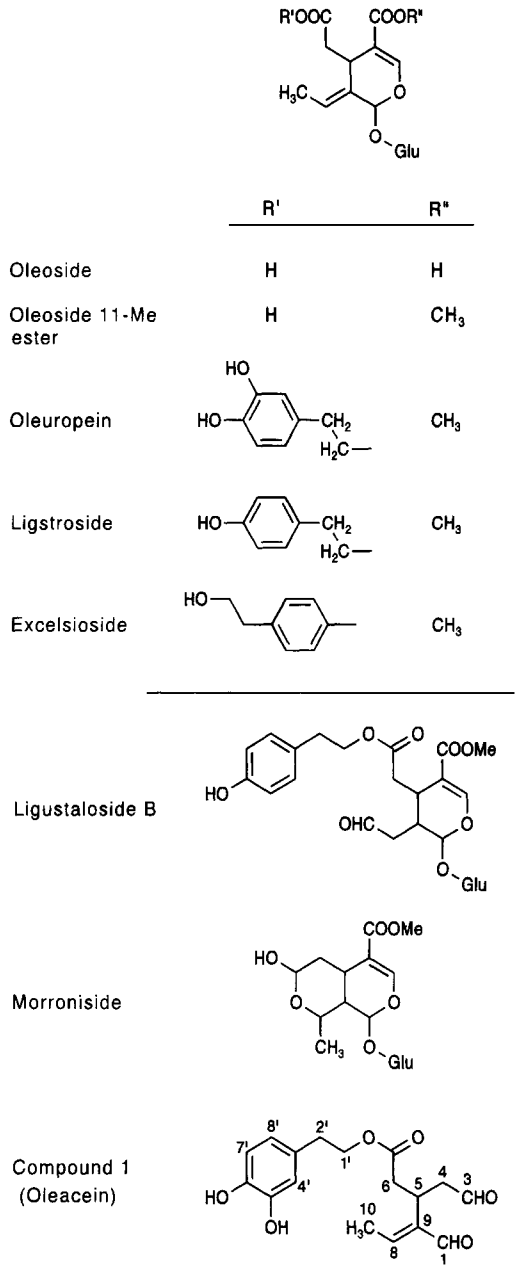
$$\log LC_{50} \pm 2\ SE\ LC_{50}$$

Results and Discussion

Oleacein *(1)* was isolated from *O. europaea* by a bioassay directed fractionation each time pursuing the most active fraction. The compound being responsible for the main ACE-inhibitory activity of *O. lancea* was localised by preparative TLC and preliminary found also to be oleacein (TLC and HPLC). Both plant extracts contain oleacein in a concentration of at least 0.06 g/l. Based on the isolation procedure from *O. europaea* a procedure for the isolation of oleacein *(1)* from *O. lancea* was made. Injecting *1* into the HPLC (detecting by scanning from 220 to 400 nm) one intense peak was eluted when using the elution program described in Materials and Methods. The NMR spectra of oleacein isolated from both plants were found to be identical to those reported by Scalzo and Scarpati (1993) and Montedoro et al. (1993), who isolated compound *1* from the juice of ripe black olives. The compound was not named, and we therefore propose the name oleacein (nomenclature derived from the words *Olea, ACE* and *in*hibitor).

The IC_{50} value of oleacein was determined to 26 µM ± 1.8 µM according to Fig. 2. In Fig. 2 n was determined to 1.83 ± 0.12. ACE is known to contain two enzymatically active sites (Soubrier et al., 1993).

When dialysing the oleacein-ACE complex for 72 hours, no reduction in ACE-inhibitory activity was observed indi-



cating that oleacein inhibits ACE irreversible or acts as a tight binding inhibitor. Oleacein acting as an irreversible inhibitor would explain the long duration of blood pressure lowering effect observed when investigating the effect of olive leaf extract in rats, cats and dogs (Janku et al., 1957; Kosak and Stern, 1959), as new ACE would have to be synthesised to restore the function of the RAS.

To investigate whether the alcohol or the secoiridoid part of oleacein is responsible for the ACE-inhibitory activity β-(3,4-dihydroxyphenyl)ethanol was synthesized by hydrolytic degradation of verbascoside. β-(3,4-dihydroxyphenyl)ethanol was recently isolated by a bioassay-directed fractionation from the olive leaves and found to be a potential calcium-antagonist (Rauwald et al., 1994). When tested for ACE-inhibitory activity β-(3,4-dihydroxyphenyl)ethanol inhibited ACE by 26% at a concentration of 0.33 mg/ml (2.1 mM) indicating that the secoiridoid part of oleacein is mainly responsible for the ACE-inhibitory activity.

The dialdehyde form of iridoid-aglycones is known to be in quilibrium with the hemiacetal form. The result indicating that the secoiridoid part of oleacein is mainly responsible for the ACE-inhibitory activity prompted us to investigate other secoiridoids and their aglycones for ACE-inhibitory activity. All the secoiridoids tested (Table 2) were found not to inhibit ACE but when enzymatically cleaved by β-glucosidase the reaction mixture displayed pronounced ACE-inhibitory activity (except for morroniside). The present results strongly imply that secoiridoid aglycones of the oleoside type generally possess ACE-inhibitory activity.

The *in vivo* toxicity of oleacein on newly hatched brine shrimp larvae (nauplii) was investigated. The results, listed in Table 1 and Fig. 3, show the typical sigmoid dose-response curve of the lethality of oleacein on nauplii at different concentrations. The acute LC_{50} (> 2400 ppm) is higher than the value reported for camphor (1880 ppm – Sam, 1993) and a chronic LC_{50} value of 969 ppm demonstrate that oleacein is relatively well tolerated by the brine shrimp larvae.

The active principles responsible for the blood pressure lowering effect of olive leaves was hitherto found to be

Table 1. Mortality of the brine shrimp larvae after 24 hours exposure to oleacein in artificial sea-water/methanol, 96.25:3.75.

Dose (ppm)	Dosage (log dose)	Accumulated Dead	alive	Accumulated dead	alive	Ratio dead/total	Mortality (%)
2400	3.38	66	2	193	2	193/195	99
1600	3.20	43	12	127	14	127/141	90
1200	3.08	38	33	84	47	84/131	64
800	2.90	25	30	46	77	46/123	37
600	2.78	14	35	21	112	21/133	16
400	2.60	7	44	7	156	7/163	4.3

Number of animals, n, was 123–195. Average number of animals, n_{av}, was 148. Column 5 (accumulated dead) is the summation of column 3 (dead) from the lowest dose down to and including any given dose. Column 6 (accumulated alive) is the summation of column 4 (alive) from the highest dose up to and including the indicated dose. LC_{50} = 969 ppm ± 1 ppm (3.03 mM ± 0.003 mM); 95% confidence limits of 901 ppm to 1042 ppm (2.81 mM to 3.25 mM).

Table 2. *In situ* enzymatic cleavage of secoiridoids and measurement of ACE-inhibition.

	ACE-Inhibition at 0.33 mg/ml		
	Glycoside	After cleavage	TES-TAPE®
Morroniside	0%	27%	positive
Oleuropein	8%	95%	positive
Ligstroside	5%	96%	positive
Excelcioside	0%	91%	positive
Oleoside 11-methyl ester	7%	64%	positive
Oleoside	16%	74%	positive
Ligustaloside B	0%	–	negative

Column 4 (TES-TAPE®) shows whether the secoiridoids were cleaved with β-glucosidase (positive) or not (negative). Ligustaloside B was neither cleaved by β-glucosidase nor β-glucoronidase (EC 3.2.1.31) from *Helix pomatia*.

oleuropein showing hypotensive, coronary dilating and antiarrhythmic action (Petkow and Manolov, 1972), and β-(3,4-dihydroxyphenyl)ethanol, found to be a potential calcium antagonist (Rauwald et al., 1994). The present study has shown that olive leaves also contain a relatively strong *in vitro* ACE-inhibitor. ACE-inhibitors and calcium antagonists in combination reduce blood pressure more than either drug alone. Various studies have highlighted the value of this powerful combination (Cappuccio and MacGregor, 1995).

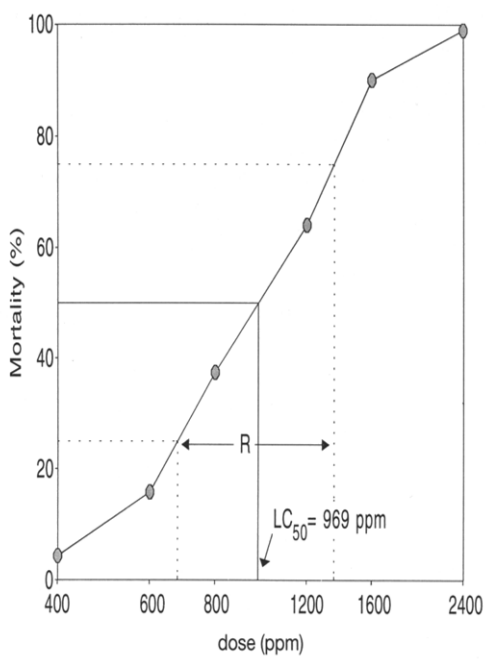


Fig. 3. Plot of concentration of oleacein *(1)* in ppm against cumulative percent mortality. LC_{50} (24 h) = 969 ppm ± 1 ppm (3.03 mM ± 0.003 mM); 95% confidence limits of 901 ppm to 1042 ppm (2.81 mM to 3.25 mM). Relative toxicity (LC_{50} (24 h) $K_2Cr_2O_7/LC_{50}$ test substance (ppm)) = 0.075. The acute LC_{50} (6 h) measured after 6 hours is higher than 2400 ppm (LC_{12} = 2400 ppm).

Combination therapy using drugs with different targets (drug groups) usually minimizes toxicity and unwanted side-effects by using each drug/active principle in low doses. A calcium antagonistic activity of oleuropein has been excluded (Rauwald et al., 1994), and oleuropein does not inhibit ACE significantly at a concentration of 0.33 mg/ml. The combination of 3 active principles with different hypotensive mechanisms might explain the reported non-toxicity of extracts from olive leaves used in the treatment of hypertension (Esdorn, 1954; Luibl, 1958). Further evidence from *in vivo* studies is needed to clarify the interactive role of the active principles of olive leaves. Future research based on bioassay directed fractionation will show whether or not further active agents influencing the blood pressure are to be found in the olive leaves.

Acknowledgements

The authors thank Birgitte Schottländer and Bente Gauguin for excellent technical assistance, Eilien Jensen for the package of HPLC column and Claus Cornett for recording the 400 MHz spectra. The Bruker AMX 400 and the Bruker AX 200 F spectrometer were acquired by means of grants from the Danish Technical Research Council, the Alfred Benzon Foundation, the Velux Foundation, I. Henriksens Foundation and the Thorkild Steenbeck Foundation.

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