Screening of Nine Vasoactive Medicinal Plants for their Possible Calcium Antagonistic Activity. Strategy of Selection and Isolation for the Active Principles of Olea europaea and Peucedanum ostruthium†

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INTRODUCTION

Calcium antagonists are in wide clinical use as therapeutical agents for the treatment of coronary heart diseases, hypertension etc. Despite this, only a few large-scale screening programmes of natural products searching for calcium-antagonistic activity in plant extracts have been established, limited to Chinese herbal medicines by Chinese and Japanese groups (Chen et al., 1988; Ichikawa et al., 1986, 1989; Yamahara et al., 1986). As a result of such investigations, naturally occurring substances with 'calcium-antagonistic' activity have been found in various groups of secondary products like alkaloids (e.g. Yano et al., 1991), coumarins (e.g. Vuorela et al., 1986), lignans (Ichikawa et al., 1986), terpenes (e.g. Hwang et al., 1987). As there are no general structure-activity relationships for these compounds (Ichikawa et al., 1989), a systematic research programme guided by literature screening for medicinal plants that are used in traditional medicine or phytotherapy seems to be sensible. The evident diversity of natural products with calcium-

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Table 1.

<table>
<thead>
<tr>
<th>Plant investigated</th>
<th>Abbreviation</th>
<th>Part used</th>
<th>Solvents used for the preparation of the extracts</th>
<th>Essential criteria for the selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guajacum officinale L.</td>
<td>GUA</td>
<td>Wood</td>
<td>Dichloromethane</td>
<td>Contains a great variety of only partly known lipophilic lignans (Steinegger and Hänsel, 1988); some lignans isolated from Articum lappa inhibited K⁺-induced contractions of aortic strips (Ichikawa et al., 1986); they have not been tested against norepinephrine-contractions yet</td>
</tr>
<tr>
<td>Cryptolepis sanguinolenta Lindley</td>
<td>CRY</td>
<td>Root</td>
<td>Ethanol 80% (raw alkaloid fraction)</td>
<td>Plant has been reported for its hypotensive activity; alkaloids are probably the active compounds (Noamesi and Bambose, 1980)</td>
</tr>
<tr>
<td>Passiflora incarnata L. (Passifloraceae)</td>
<td>PASS</td>
<td>Herb</td>
<td>Ethanol 60% chloroform</td>
<td>Plant is reported to have spasmylocytic (Lutomski et al., 1981) and hypotensive properties and to increase coronary flow (Ambühl, 1966)</td>
</tr>
<tr>
<td>Ammi visnaga (L.) Lam. (Apiaceae)</td>
<td>AMMI</td>
<td>Fruit</td>
<td>Methanol chloroform</td>
<td>Ammi is well known for its spasmylocytic and slightly antihypertensive properties, although heline and visnadin can be found in pharmaceutical formulations their mode of action is not quite clear.</td>
</tr>
<tr>
<td>Solidago gigantea Ait. (Solidaginaceae)</td>
<td>SOL</td>
<td>Herb</td>
<td>Hot water chloroform</td>
<td>Watery extracts have a hypotensive activity (Rácz et al., 1980), diluted alcoholic extracts might have spasmylocytic properties (Westendorf and Vahlensieck, 1981)</td>
</tr>
<tr>
<td>Ruta graveolens L. (Rutaceae)</td>
<td>RUT</td>
<td>Root</td>
<td>Dichloromethane</td>
<td>Contains spasmylocytic compounds (Pétill-Paly et al., 1982)</td>
</tr>
<tr>
<td>Olea europaea L. (Oleaceae)</td>
<td>OLEA</td>
<td>Leaf</td>
<td>Ethanol 30%</td>
<td>Diluted alcoholic extracts are spasmylocytic, antihisrhythm and hypotensive (Petkov, 1979) and influence monophasic action potential in anaesthetised dogs (Occhiuto et al., 1990)</td>
</tr>
<tr>
<td>Peucedanum ostruthium (L.) Koch</td>
<td>PEU</td>
<td>Root</td>
<td>n-Hexane methanol</td>
<td>In some Peucedanum species furanocoumarins with calcium-channel blocking activity have been found (Vuorela et al., 1986); apart from such compounds, P. ostruthium contains coumarins that have not been investigated yet German name ‘Hergespannkraut’, spasmylocytic.</td>
</tr>
<tr>
<td>Leonurus cardiaca L. (Lamiaceae)</td>
<td>LEO</td>
<td>Herb</td>
<td>Methanol 50% chloroform</td>
<td>Antihypertensive and negative inotropic properties have been reported (Reuter and Diehl, 1970) but have not been confirmed on a phytochemical basis (Wichtl, 1990)</td>
</tr>
</tbody>
</table>

operated-channels (ROC). Calcium-channel-blockers like verapamil or diltiazem only inhibit K⁺-induced contractions since they have only weak affinity to ROC. Therefore, a comparison of these two methods can give information about the selectivity of the tested compounds.

We report a first systematic screening of 14 different extracts from nine medicinal plants for their possible calcium antagonistic activity. Based upon this screening, the active principles of Olea europaea leaves and Peucedanum ostruthium rhizomes were isolated, identified and compared with regard to the selectivity tests. At the beginning the different pharmaceutical and medicinal criteria are described, according to which these medicinal plants used in European and African phytotherapy and traditional medicine were chosen.

Preparation of the extracts. 50 g of the dried, milled plant parts was stirred for 20 min under reflux with 200 ml of the solvents named in Table 1 at a temperature that did not exceed 50°C. After filtration of the mixture, this extraction procedure was repeated twice to obtain optimal yields. The combined solvents were evaporated in vacuo and the dry or oily extracts were stored cold and dark.

Isolation and identification of the active principle from Olea europaea leaves. 5 g of the Olea extract was dissolved in warm methanol and fractionated by CC using 220 g of silica gel (0.043–0.06 mm, Macherey-Nagel, Düren). As eluent, chloroform with increasing amounts of methanol was used (95:5, 80:20, 50:50, methanol 100%). The elution was controlled by TLC using ethylacetate–dioxane–water 30:10:0.3 as the mobile phase. The collected fractions were combined according to TLC similarities resulting in five raw fractions (O1–O5). The pharmacologically active fraction O1 (see Fig. 3) was dissolved in 3 ml of chloroform and rechromatographed by CC eluting with chloroform–methanol mixtures (98:2, 95:5, 90:10). As a result of this procedure, fraction O1 gave one single spot on TLC (Rf-value: 0.8; solvent as mentioned above). Final purification was carried out by CC using Sephadex LH20 (Pharmacia, Uppsala, Sweden) as the stationary and methanol as the mobile phase. O1 was identified as 3,4-dihydroxyphenylethanol by TLC comparison (mobile phase as mentioned above, detection:

MATERIAL AND METHODS

Plant material. The plants listed in Table 1 were purchased as dried plant parts (Fa. Caelo, Hilden, FRG) except for Cryptolepis roots which were obtained milled from the Centre for Scientific Research into Plant Medicine (Ministry of Health), Mampong-Akwamip; Ghana. A herbarium specimen of Cryptolepis is located at the Faculty of Pharmacy, University of Science and Technology, Kumasi, Ghana. Voucher specimens of the other plants are deposited at the Institute of Pharmaceutical Biology.
Fe(III)Cl₃ with the authentic alcohol obtained by hydrolytic degradation of oleanoic acid. The optimal conditions of acid hydrolysis were: 1 M H₂SO₄, 100°C, 1 h; the reaction mixture was neutralized by adding NaHCO₃ and extracted with ethyl acetate. The EI-MS of this alcohol showed the molecular ion m/z 154 [M⁺, 28%] and the base peak m/z 123 [100%, C₇H₁₄O₂] due to a characteristic fragmentation of primary alcohols (Spiteller, 1966).

Oleanoic acid was identified as the main component of fraction O3 by TLC comparison (Rf-value: 0.3; solvent as mentioned above) with authentic substance isolated according to DAC (1972) and purified by CC using silica gel as stationary phase and chloroform-methanol mixtures as eluent; the more polar fractions O4 and O5 contained various unidentified enolic acid type compounds (Brehm, 1993).

HPLC analysis of 3,4-dihydroxyphenylethanol. The purity of the isolated compound was determined as at least 98% by a new HPLC method using methanol 50% + H₃PO₄ 0.5% (V/V) as mobile phase and Lichrospher RP18, 5µm (Merck, Darmstadt) as stationary phase; Gynkotech delivery system 480, Waters multiwavelength detector 490-MS, GynkoSoft chromatography data system, version 2.62 (Brehm, 1993).

Figure 1. Isolation scheme for Peucedanum ostruthium.

Isolation and identification of the active principles from Peucedanum ostruthium rhizomes (see also Fig. 1). Since the n-hexane extract showed the best activity in the pharmacological pre-tests using different solvents (Fig. 4), it was fractionated by CC using silica gel (0.04–0.063 mm, Macherey-Nagel, Düren) and a mixture of cyclohexane-tetrahydrofuran-isopropanol (88:11:1) until the striking blue fluorescent ostruthin (Wagner el. al., 1982) could be detected by TLC (Rf-value: 0.55; toluene-diethylether-acetic acid 10% (1:1:1), upper layer). For further elution, cyclohexane-tetrahydrofuran-isopropanol (75:23:2) was used as an eluent in which the compounds A, B, C were purified by crystallization. D, E, F were separated by reversed-phase MPLC: LiChroprep RP 18, 25–40 µm (Merck, Darmstadt) as stationary phase and methanol–water (60:40) as mobile phase; Büchi 681 Chromatography Pump. While the compounds D and F could be obtained pure with this procedure, E had to be purified by repeated CC and preparative TLC using cyclohexane–tetrahydrofuran-isopropanol 75:23:2 as mobile phase. Compound B was identified as imperatorin by comparison with authentic substance, compound D as ostruthol and compound E as 2''-O-acetyl-oxypeucedanin hydrate (5-[2-acetoxy)-3-hydroxy-3-methyl-butoxy]psoralen), both by one- and two-dimensional ¹H/¹C NMR spectroscopy (Brehm, 1993).

The three compounds A, C, F with weaker pharmacological activity in our first survey (see Fig. 5) were identified by comparison with literature data as isoimperatorin (A), ostruthin (C) and oxypeucedanin hydrate (F), all known furanocoumarins in Peucedanum ostruthium.

Pharmacological testing. The descending thoracic aorta of rabbits of either sex was excised and dissected free of connective tissue. The aorta was cut into 2 mm wide ring segments and cut off into small strips. These were suspended in organ baths (25 ml) containing Krebs-Henseleit solution at 37°C degassed with 5% CO₂ in O₂ (pH 7.4). The suspended strips were stretched with a load of 2 g and contractions were recorded isometrically with electromechanical transducers and a potentiometric recorder. After equilibration the strips were contracted with 0.5 ml of a KCl solution, the final concentration in the bath being 60 mm. When the contractions had reached a stable maximum, they were washed out and allowed to relax to baseline levels. The addition of KCl was repeated. Strips that did not give reproducible recordings or did not relax after washing out were not used. The extracts were dissolved in dimethylsulphoxide (DMSO, 0.1 ml per bath) and added to the bath solution onto the plateau. The final concentration of the extracts was 10⁻³ g/ml or 10⁻⁴ g/ml respectively; the latter concentration was used in case of solubility problems. Extracts that had no significant activity at these high concentrations have not been examined further. Pure DMSO (0.1 ml in 25 ml bath solution) hardly influenced the contraction. The relaxation was measured 15 min after addition. All values reported represent the mean of 4–10 individual experiments. The significance between
the mean values was determined by Student's t-test (Olea) and Mann--Whitney test (Peucedanum) (*p<0.05, **p<0.01). In the following, the completed data only of ostruthol (D) are given; for further coumarins, for 3,4-dihydroxyphenylethanol and for the solvent see Rauwald et al. (1991). Ostruthol at concentrations of 0, 31.6, 100, 316 ng/ml gave heights of KCl-(60 mM)-contraction (mm) of 23.3±7.83, 14.3±6.07, 8.7±3.70, 5.6±3.10, respectively. The heights of norepinephrine-(100 µM)-contraction (mm) were 23.7±6.91, 20.6±6.52, 17.6±5.91, 14.3±2.58, respectively.

RESULTS AND DISCUSSION

The nine plants investigated were selected after a literature screening for their use in traditional medicine or phytotherapy and are listed in Table 1. They were chosen according to the following general criteria: use in traditional medicine in the treatment of cardiovascular diseases; 'nonspecific' spasmolytic activity (mode of action not known); natural compounds with structural similarities to already known 'calcium-antagonists'; calcium-antagonists that have been found in species of the same tribe. The results of our pharmacological screening using the inhibition of aortic contractions induced by K+ -depolarization are shown in Fig. 2: extracts derived from Ammi visnaga, Guajacum officinale, Olea europaea, Peucedanum ostruthium and Ruta graveolens showed a clear activity, while Cryptolepis sanguinolenta, Leonurus cardiaca, Passiflora incarnata and Solidago gigantea had no activity in this test. The criteria in determining the best solvents for the extraction were the extracts' biological activity, as demonstrated for Peucedanum in Fig. 4, and data in the literature about pharmacologically active compounds and their polarity. The results obtained with Leonurus, Solidago and Passiflora were confirmed by an additional investigation of their lipophilic extracts, since most calcium antagonists have lipophilic character. The increased K+ -contraction observed for Leonurus and Solidago extracts may be due to the occurrence of membrane damaging saponins.

A bioassay guided isolation strategy for the active principle of the Olea extract resulted in five fractions O1–O5, which were again tested against K+-induced aortic contractions (see Fig. 3). The most active O1 consisted of 3,4-dihydroxyphenylethanol which was identified by chromatographic and spectroscopic methods. Neither oleuropein, the main constituent of O3, nor the enolic acid type-containing fractions O4/O5 showed any clear inhibition. Thus, for oleuropein, which has been reported to be the active spasmolytic and antihypertensive principle in Olea europaea (Hän sel and Haas, 1984), a calcium antagonistic activity can be excluded. 3,4-Dihydroxyphenylethanol is the esterifying alcoholic component and a degradation product of oleuropein (see formula). It is found in dried leaves and in certain commercial dry extracts, possibly due to the known instability of oleuropein (Huber, 1975; own results unpublished). As the inhibition of contractions induced by K+-depolarization does not necessarily indicate a calcium channel blocking mode of action, the purified alcohol was also tested against norepinephrine-induced contractions in a preliminary pharmacological investigation (Rauwald et al., 1991). It exhibited no clear selectivity but showed in both tests similar activities (Fig. 6). Therefore, a selective blockade of the VOC can be excluded; other modes of action may be...
involved in the spasmylic activity, e.g. 'nonselective' calcium antagonists like cinnarizine also inhibit norepinephrine-induced contractions (Godfraind and Kaba, 1972). Nevertheless, a possible differentiation between oleuropein and its alcoholic component concerning the mode of action is reported in this paper for the first time. These results may coincide with Occhiuto et al. (1990) and Zarzuelo et al. (1991) who reported in their recent pharmacological investigations on Olea europaea that, in addition to oleuropein, at least one other active compound must be present in this drug.

The pharmacologically most active extract of Peucedanum rhizomes was obtained by using n-hexane as solvent (Fig. 4). Therefore, this extract was fractionated by a combination of CC resulting in six pure, crystalline coumarins which were identified as isoimperatorin (A), imperatorin (B), ostruthin (C), ostruthol (D), 5-[2-acetoxy]-3-hydroxy-3-methylbutoxypсорalen (E) and oxypeucedanin hydrate (F) (Fig. 1). E has been reported once as a minor compound of Ammi majus (Ivie, 1978) and is thus new in Peucedanum ostruthium. To establish their activity profiles, these compounds were tested against K⁺-induced contractions in a pharmacological survey at a concentration that allowed a comparison of their activity as shown in Fig. 5. The three most active substances B, D, and E were chosen for a further, more detailed pharmacological study in which the compounds' activity at various concentrations as well as their selectivity in comparison with norepinephrine-induced contractions were investigated (Rauwald et al., 1991). The most active compound ostruthol has in the meantime been tested more comprehensively and the results of this procedure are shown in Fig. 6. It exhibits a much higher activity against K⁺-spasms than against norepinephrine-contractions, suggesting a blockade of voltage-operated calcium channels.

These results also enable a differentiation between ostruthol as a compound that behaves similarly to the selective calcium channel blocker verapamil also tested in this model, although to a lesser degree (Brell, 1993), and 3,4-dihydroxyphenylethanol as a nonselective compound, which shows no differentiating pharmacological action in these tests but exerts a nonselective activity as, for example, described for papaverine (Weishaar et al., 1983).

If we compare the pharmacological data of ostruthol and verapamil, the relative potency of ostruthol can be estimated at approximately one-tenth to one-hundredth of that of verapamil in our tests. This is the same order of magnitude as the already classified naturally occurring calcium antagonists like lignans from Magnolia fargesii (Chen et al., 1988) or a sesquiterpene from Tussilago farfara (Hwang et al., 1987). Although this testing model can differentiate between selective and nonselective compounds as well as between calcium antagonists and alpha-receptor blockers (Weishaar et al., 1983), one must be aware of the fact that, beside these two testings, a number of pharmacological investigations like ligand binding studies, myocardial contractility studies or investigations concerning the tissue selectivity are necessary to characterize the pharmacological profile of a calcium antagonist. A direct and ultimate way to classify the mode of action of various channel blockers are electrophysiological methods like patch-clamp techniques which are still difficult to handle (Spedding and Cavero, 1984) and therefore too elaborate for a large scale screening of raw extracts. Although calcium antagonism may not be the only pharmacological mode of action that is involved in pharmacology of ostruthol, we are convinced that such investigations can promote our knowledge of the pharmacology of natural products and contribute to a rational judgement of activity or lack of activity of medicinal plants used in phytotherapy.

Figure 5. Inhibition of K⁺-induced aortic contractions by six crystalline Peucedanum coumarins A–F isolated from the pharmacologically active n-hexane extract, concentration 0.01 mg/mL.

Figure 6. Evaluation of activity and selectivity of 3,4-dihydroxyphenylethanol from Olea leaves (left) and ostruthol from Peucedanum rhizomes (right) by comparison between K⁺- and norepinephrine-induced spasms.
REFERENCES


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