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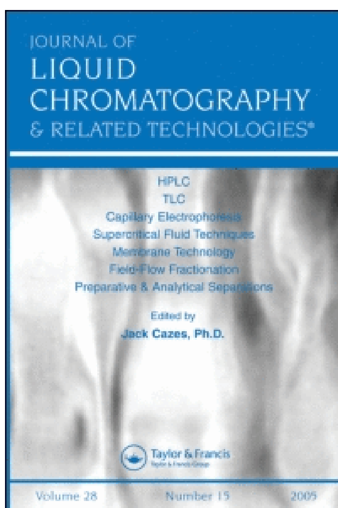

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APPLICATION OF CENTRIFUGAL PARTITION CHROMATOGRAPHY IN A GENERAL SEPARATION AND DEREPLICATION PROCEDURE FOR PLANT EXTRACTS

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ABSTRACT

Centrifugal Partition Chromatography (CPC) was used in a general separation and dereplication procedure in search for new biologically active compounds from crude plant extracts. In this procedure, the alcoholic extract was prefractionated by CPC with the solvent system heptane/ethyl acetate/methanol/water 6:1:6:1 (v/v/v/v) followed by bioactivity screening of the fractions. The active fractions were analyzed for the known active components for dereplication. If the activity was found in the most polar fraction, which tended to contain a large group of compounds, the fraction was separated again by CPC (solvent system ethyl acetate/methanol/water 43:22:35, v/v/v). This two-step procedure was found to be efficient for five extracts tested for both chemical and bioactivity profiles.

INTRODUCTION

The use of centrifugal partition chromatography (CPC) for natural product research has been increasing since the last decade. Like any other countercur-

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rent liquid-liquid partitioning chromatography, CPC has the advantage of a great many choices of solvent systems, high recovery of the sample and relatively mild chromatographic conditions. Moreover, CPC offers shorter running times compared to conventional countercurrent chromatography. It has high loading capacity and it allows a relatively easy scale-up. This chromatographic technique was described for the first time by Murayama.[1] Theoretical and application aspects were extensively discussed by Foucault.[2]

In a previous study, we reported the development of a CPC prefractionation step of plant extracts prior to bioactivity screening. The solvent system heptane/ethyl acetate/ methanol/ water 6:1:6:1 (v/v/v/v) was selected as most suitable.[3,4,5,6] By means of this reproducible prefractionation, the fractions containing compounds that give false-positive reactions or that interfere with the bioassays used can be identified in an early stage.[4] The chance of finding leads among minor compounds is also increased.

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In the present study, the CPC prefractionation process was evaluated for its efficiency to separate bioactive compounds from plant ethanol extracts. Four different bioassays, i.e., radioligand binding assays for adenosine A_1 and opiate receptors, a microplate assay for acetylcholinesterase (AChE) inhibitory activity, and an anti-asthmatic assay were used. Seven ethanol extracts from different plant families with supposedly different bioactivities were fractionated by CPC and the fractions were tested for bioactivities. *Narcissus* spp. (Amaryllidaceae) are known to contain the alkaloid galanthamine, which can inhibit AChE and also has opioid activity.[7] Cocoa beans (*Theobroma cacao* L., Malvaceae) contain theobromine and caffeine which are well-known antagonists for adenosine A_1 receptors. *Senna* spp. (Fabaceae) is known to contain flavonoids which might show an activity on the same receptor. Three medicinal plants from Indonesia, i.e., *Eucalyptus globulus* Labill. (Myrtaceae), *Vitex trifolia* L. (Verbenaceae), and *Orthosiphon stamineus* Benth (Lamiaceae) were chosen to be tested for anti-asthmatic activity based on their traditional usages.

For all extracts studied, the most active fraction, excluding the false positives,[4] was the fraction that contains the majority of the polar compounds. It, thus seems that, also, a general applicable method should be developed for the second separation step. The "best solvent" approach as reported by Foucault[2] was applied to select suitable CPC solvent systems. The fractions from five of the extracts were used in these studies. By comparing the partitioning of compounds over the two phases of potentially suitable CPC solvent systems selected by the "best solvent" method, a CPC system was selected. Using this system as a second step, the efficiency of the whole two-step procedure was evaluated by the separation of the components detected by TLC and the distribution of bioactivity over the subfractions obtained.

EXPERIMENTAL

Chemicals

The reference phenolic compounds were a gift from Mr. L. C. Verhagen, Heineken Technical Services, Zoeterwoude, The Netherlands, unless otherwise stated. Linoleic acid, tannic acid, acetylthiocholine (ATCI), 5, 5'-dithiobis-[2-nitrobenzoic acid] (DTNB), bovine serum albumin (BSA), and acetyl-cholinesterase (AChE) were obtained from Sigma (St. Louis, MO, USA). Quercetin was obtained from Fluka (Buchs, Switzerland). Anisaldehyde was purchased from Acros Organic (New Jersey, USA), the radioligands were obtained from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). N^6-cyclopentyl adenosine (CPA) was obtained from RBI (Natick, MA, USA). Morphine was purchased from ACF Chemiefarma NV (Maarsen, The Netherlands). All organic solvents (analytical-reagent grade) were purchased from J. T. Baker (Deventer, The Netherlands). For the microplate assay for AChE inhibitors, 50 mM Tris-HCl pH 8.0 was used as a buffer through out the experiments unless otherwise stated. AChE was from an electric eel (type VI-S lyophilized powder, 480 U/mg solid, 530 U/mg protein). The lyophilized enzyme was dissolved in buffer to obtain a 1130 U/mL stock solution. Further enzyme dilution was obtained by dissolving in 0.1% BSA in buffer. DTNB was dissolved in buffer to which 0.1 M NaCl and 0.02 M $MgCl_2$ were added. ATCI was dissolved in millipore water.

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Plant Material

The flowers of *Senna siamea* (Lam.) Irwin & Barneby (Fabaceae) (syn. *Cassia siamea* Lam.) were collected in Phitsanulok, Thailand in July 1998. A voucher specimen is deposited at the department of Medicinal Chemistry and Pharmacognosy, Naresuan University, Phitsanulok, Thailand. The bulbs of *Narcissus* 'Carlton' and *Narcissus* 'Sir Winston Churchill' (Amaryllidaceae) were obtained from W.F. Leenen & Zn., Sassenheim, The Netherlands. *Theobroma cacao* L. (Malvaceae) (syn Sterculiaceae) beans from Ghana were obtained from A.D.M. Cocoa B.V., The Netherlands. The leaves of *Eucalyptus globulus* Labill. (Myrtaceae), *Vitex trifolia* L. (Verbenaceae), and *Orthosiphon stamineus* Benth (Lamiaceae) were collected from Yogyakarta, Indonesia. The voucher specimens are kept at Gadjah Mada University, Yogyakarta, Indonesia.

Extraction

Dried plant material was cut into small pieces and macerated with 5 mL ethanol per gram for 4-7 days. The suspension was then filtered and the filtrate was evaporated to dryness under reduced pressure.

CPC Apparatus

A modular Sanki (Kyoto, Japan) centrifugal partition chromatograph (type LLN) was used. It consisted of a power supply (Model SPL), a triple-head constant-flow pump (Model LBP-V) and a centrifuge (Model NMF). The centrifuge can contain up to 12 cartridges with a total volume of 250 mL. A Panasonic Pen-recorder (Model VP 67222A) was connected to a UVIS 200 detector (Linear Instruments, Reno, NV, USA). Fractions were collected by means of a LKB 2211 Superrac fraction collector. In all experiments, six cartridges (total internal volume 125 mL) were used. The pressure was limited to a maximum of 60 bar. The flow rate was set to 2 mL/min. The size of the subfractions collected was 8 mL.

The First Separation Step by CPC (CPC-1)

The solvent system used was heptane/ethyl acetate/methanol/water 6:1:6:1 (v/v/v/v). The CPC was used in the ascending mode; the organic phase was used as mobile phase and the aqueous phase was used as stationary phase. The sample was dissolved in 4 mL of each of the two phases before injection. Twenty subfractions (160 mL including the void volume) were collected. As the void volume of CPC-1 was approximately 45 mL, subfractions 1-5 were discarded. After subfraction 20, the mobile phase was changed from the organic phase to the aqueous phase without changing the direction of the run. Theoretically, all sample components would be eluted in 125 mL, which was the internal volume in the CPC apparatus, and fraction 36 was supposed to be the last fraction. However, we collected the eluate until fraction 40 to be certain that no samples remained in CPC. In this way, a CPC run took less than 3 hours and the CPC was ready for the next run as it had been refilled by the stationary phase.

All subfractions were then concentrated and analyzed by means of TLC. Subfractions that showed similar chromatograms were pooled and dried under reduced pressure. After pooling, all fractions obtained from this step were tested for bioactivity.

Development of the Second Separation Step (CPC-2)

Search for the Best Solvent for the Polar Fraction from CPC-1

A series of solvents was tested and the solvent that could dissolve 500 μg of the fraction of interest from CPC-1 with the smallest volume was selected as the best solvent.

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Test for the Best CPC Two-Phase System

Some CPC solvent systems containing the best solvent as an intermediate polar solvent were selected. They were prepared in three different solvent-ratios based on their ternary diagrams.[2] For each solvent system, 50 μL of lower phase and 50 μL of upper phase were added to a microtube containing approximately 2-3 mg of dried fraction of interest from CPC 1. The tubes were shaken vigorously on a shaker for 30 min. Then the two phases were separated and dried under vacuum. The partitioning over both phases was analyzed by means of TLC.

The spots observed on TLC for the upper phase and lower phase of each two-phase solvent system were counted. Spots were grouped in Rf<0.5 and Rf>0.5. All spots that could be detected by UV 254, UV 366, and modified anisaldehyde-sulphuric acid spray reagent[8] were counted. Partially overlapping spots were counted separately. By this method each solvent system can be represented by 4 scores, e.g.:

No. of spots in upper phase with: Rf > 0.5 (A)

Rf < 0.5 (B)

No. of spots in lower phase with: Rf > 0.5 (C)

Rf < 0.5 (D)

To simulate the orientation on the TLC-plate these scores are represented as shown in Figure 2.

As the separation by CPC is based on partitioning of the sample's components between two phases, a good solvent system should show an almost equal score for upper and lower phase in this evaluation method. A rating system for the selection of the most suitable solvent system was based on the following consideration. The more corresponding spots are present in both phases, the better the CPC separation will be (assuming that each compound has different partition coefficients). Comparison of the numbers of compounds with Rf>0.5 and those with Rf<0.5 gives information about polarity of the compounds present. A suitable two-phase system should give a good distribution between both polar and non-polar compounds in both phases, i. e., each quadrant of Figure 2 should contain a series of compounds. According to this, some two-phase systems were discarded by the following criteria:

Score in A or B or C or D = 0.

Scores in A and D or B and C =1.

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Score in A and B or C and D =1.

From five extracts tested, the solvent system that showed the best potential in this evaluation method was chosen and used in the second CPC fractionation (CPC-2).

The Second CPC Fractionation (CPC-2)

The selected solvent system: ethyl acetate/methanol/water 43:22:35 (v/v/v) was prepared and allowed to be equilibrated for 24 hr. Ascending mode was used in CPC. Twenty subfractions (160 mL including the void volume) were collected. Then in the same mode of elution, the mobile phase was changed from organic phase to aqueous phase to collect another 160 mL. Subfractions that showed similar TLC chromatograms were combined.

Evaluation of the Separation by CPC-2

The separations of the most polar fractions of five plant extracts by CPC-2 were evaluated by means of TLC as described before.[3] This evaluation method is based on the number of compounds detected by TLC per subfraction. The more subfractions with a low number of compounds, the higher the efficiency of the separation. The distribution of the biological activity over the fractions was also examined.

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TLC Analyses

Samples were applied on 20x10 cm silica gel plates F254 No. 5554 (Merck, Darmstadt, Germany) and developed in saturated TLC chambers which were pre-equilibrated for about 30 min. For all experiments, two TLC solvent systems, i.e., chloroform/methanol 9:1 (v/v) and ethyl acetate/formic acid/acetic acid/water 100:11:11:27 (v/v/v/v) were used. Visual detection was done at 254 and 366 nm. Then the TLC plate was sprayed by modified anisaldehyde-sulphuric acid spray reagent.[8] After spraying, the plates were heated with a hot air blower for 2 minutes. Color changes after heating were noted. For the analyses of phenolic compounds, solvent system chloroform/methanol/ acetic acid 60:40:0.5 (v/v/v) was used as the third TLC system.

Radioligand Receptor Binding Assays

The adenosine A_1 receptor and the opiate receptor binding assays were carried out on cortical membranes from rat brains. Membranes were prepared according to the method of Lohse et al.,[9] except that the membranes were incubated with 2 IU/mL adenosine deaminase at 37°C before storage, as described

by Pirovano et al.[10] Protein concentrations were measured with the bicinchonic acid method.[11]

The adenosine A_1 receptor binding assays were performed with 0.4 nM [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) as the radioligand (K_d 0.39 nM). The assays were performed as originally described by Lohse et al.[12] Non-specific binding was determined in the presence of 10^{-5} M N^6-cyclopentyladenosine (CPA).

The opiate receptor binding assays were performed as described by Cox[13] using 1.5 nM [^{3}H]naloxone as the radioligand (K_d 2.1 nM). Non-specific binding was determined in the presence of 10^{-5} M morphine.

A Microplate Assay for AChE Inhibitory Activity

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The assay of AChE inhibitory activity for a 96-well microplate reader was modified from the assay of Ellman et al.[14] 125 µL of 3 mM DTNB, 25 µL of 15 mM ATCI, and 50 µL of buffer were added to the wells followed by 25 µL of sample dissolved in buffer. The microplate was then read by a Bio-Rad microplate reader model 3550 UV (Bio-Rad laboratories, Richmond, CA) at 405 nm every 13 sec for 5 times. Then, 25 µL of 0.226 U/mL AChE solution was added to the wells and the microplate was read again at the same wavelength every 13 sec for 8 times. The absorbance measured was linear for more than 2 min. The velocities of the reactions before and after adding enzyme were calculated by a Microplate Manager software version 4.0 (Bio-Rad laboratories). The results were corrected for spontaneous hydrolysis of the substrates. Enzyme activity was calculated as a percentage compared to an assay using buffer without any inhibitor.

Anti-Asthmatic Assay (in Vitro Inhibition of Antigen-Induced Contraction of Guinea Pig Trachea)

Male Hartley guinea pigs (650-750 g) were given, via i.p. injection, 3 mg ovalbumin in 300 µL saline (0.9% NaCl). This sensitization procedure was performed at least 2 weeks before use. The assay was done based on the method described by Zhang et al.[15]

RESULTS AND DISCUSSION

Evaluation of CPC-1

In a previous study, we described how CPC-1 could be used for separation of active compounds from plant extracts. In the present study, this approach was further validated using some plant extracts containing known active com-

Table 1

The Active Fraction from the Separation of Some Ethanol Extracts by CPC-1

Crude Plant Extracts	Active Fractions, Presented in No. of Subfractions Pooled			
	Adenosine A_1 Receptor Binding Assay[a]	Opiate Receptor Binding Assay[a]	Assay for AChE Inhibitory Activity[b]	Anti Anti-Asthmatic Assay[c]
Eucalyptus globulus	nd[e]	nd	nd	35-36
Narcissus 'Carlton'	9-21	--[d]	33-36	nd
Narcissus 'Sir Winston Churchill'	9-21	--	35-36	nd
Orthosiphon stamineus	nd	nd	nd	35-36
Senna siamea	9-21	9-21	--	nd
Theobroma cacao	9-21, 35-36	--	--	nd
Vitex trifolia	nd	nd	nd	35-36

[a] Fractions that show less than 40% specific radioligand remaining in the radioligand binding assays at a concentration of 0.1 mg/mL. [b] Fractions that show less than 40% of AChE activity at a concentration of 0.1 mg/mL. [c] Fractions that could inhibit the spasmodic effect of 5 µg/mL ovalbumin at a concentration of 50 ng/mL. [d] Not active. [e] Not determined.

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pounds, as well as, some plant extracts known to exhibit a certain activity. The aim was to see if rapid dereplication is feasible by using CPC-1 and to develop a strategy for the second separation step.

Seven ethanolic extracts were separated by CPC-1 and the subfractions obtained were pooled according to the similarity from TLC analyses. The bioactivities of the pooled fractions were determined (Table 1). For most extracts, the pooled fraction from subfractions 35 and 36, which was the most polar fraction, showed high bioactivity in the assays tested. These fractions from both *Narcissus* cultivars gave high inhibitory activity on the AChE assay and the same fraction from *T. cacao* showed activity on the adenosine A_1 receptor binding assay.

By comparison with the reference compounds on TLC, galanthamine was found in this most polar fraction from *Narcissus* 'Carlton' while theobromine and caffeine were found in the same polar fraction from *T. cacao.* Moreover, the anti-asthmatic activity was also found in the same fraction from the three

plant extracts from Indonesia. Subfractions 9-21 of most extracts showed high activity on the adenosine A_1 receptors and somewhat less on the opiate receptors. TLC analyses of these subfractions showed the presence of fatty acids which are known for their positive effects for some radioligand binding assays.[4]

The non-selective activity of fatty acids was thus rapidly recognized in this procedure. However, all known active compounds as well as most of the activity was found in a single fraction. The question was raised whether other known non-selective compounds such as phenolic compounds would come out in a separate fraction. Therefore, some phenolic reference compounds and linoleic acid were injected into CPC-1 to determine in which fractions they would be eluted. While linoleic acid was obtained in subfraction 9-21, all phenolic compounds were eluted together in subfractions 35 and 36 (Figure 1). The subfractions also contained all activity in the present study, as well as in our previous studies, where the alkaloids and the flavonoid that showed bioactivities on opiate and adenosine A_1 receptor binding assays respectively, were found.[5,6] It thus became clear that although CPC-1 could concentrate the bioactivity into certain fractions, a further general separation method for this polar fraction could be very useful for dereplication.

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Reference compounds	**Fractions from CPC-1**																														
	6	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**
caffeic acid																													■	■	
catechin																													■	■	
epicathe-chin																													■	■	
gallic acid																													■	■	
linoleic acid				■	■	■	■	■	■	■	■	■	■	■	■	■															
quercetin																													■	■	
quercitrin																													■	■	
rutin																													■	■	
syringic acid																													■	■	
tannic acid																													■	■	

Figure 1. The retention of some reference compounds when the mixture of 10 mg each was injected in CPC-1.

Table 2

Evaluation of Best Solvents by the Smallest Volume that Can Dissolve 500 μg of the Most Polar Fraction from CPC-1

Solvent	The Smallest Volume to Dissolve Sample (μL)				
	SS[a]	NT	EG	VT	OS
Acetone	>>[b]	>>	50	1000	>>
Acetonitrile	>>	>>	nd[c]	nd	nd
1-Butanol	>>	>>	100	>>	>>
Chloroform	>>	>>	20	1000	>>
Ethanol	~ 1000	~ 1000	100	400	200
Methanol	20	20	300	50	20
1-Propanol	>>	>>	100	300	>>

[a] Samples are the most polar fractions from: SS = *Senna siamea,* NT = *Narcissus* 'Sir Winston Churchill', EG = *Eucalyptus globulus,* VT = *Vitex trifolia*, OS = *Orthosiphon stamineus.* [b] >> = not completely dissolved in 1 mL of this solvent. [c] nd = not determined.

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Selection of a Two-Phase System for the Second Separation Step by CPC (CPC-2)

As the sample of interest was always the most polar fraction obtained from the first separation step, it contained compounds with the same range of polarities. One way to select a CPC solvent system is to find the "best solvent" that can dissolve the sample and then choose a two-phase system for CPC separation using the best solvent as an intermediate polar solvent.[2] The most polar fraction, the pool of subfraction 35-36, of five extracts from CPC-1 were tested for the best solvent (Table 2). From seven solvents tested, methanol was found to be the best solvent.

Four two-phase systems were selected as possibly suitable solvent systems for the extracts according to ternary diagrams[2] (Figure 2). They were prepared in three different ratios and then tested by dissolving the fractions of interest in the upper and lower phases. They were evaluated by the method described in Experimental. When comparing the results from the five extracts, the best two-phase system in this evaluation method was ethyl acetate/methanol/water as for the three ratios of this two-phase system, the non-suitability was found only twice. The butanol/methanol/water system was the second best but the high boiling point of butanol made it less useful for the preparative scale separation. The experiment suggested that the ethyl acetate/methanol/water system showed

solvent system[a]	ratio (v/v/v)	number of the spots detected from TLC[b]: SS[c]	NT	EG	VT	OS
$CHCl_3/MeOH/H_2O$	50/10/40	[4 2 / 4 0]	3 2 / 6 1	[1 3 / 4 1]	2 5 / 2 1	2 3 / 3 2
	50/20/30	4 2 / 5 1	4 3 / 7 4	2 4 / 3 1	3 5 / 2 1	2 3 / 3 2
	40/35/25	5 3 / 5 2	2 1 / 5 2	[1 5 / 2 1]	[3 5 / 2 0]	2 3 / 3 2
1,2 dichloroethane/ $MeOH/H_2O$	50/8/42	[5 1 / 5 1]	4 3 / 7 2	[1 4 / 2 0]	[0 4 / 2 1]	2 3 / 3 2
	50/24/26	5 3 / 5 2	3 1 / 8 2	3 4 / 5 1	[1 4 / 2 1]	2 3 / 3 1
	50/32/18	5 2 / 4 1	[3 1 / 7 1]	[1 3 / 2 1]	4 5 / 2 1	3 3 / 3 2
$EtOAc/MeOH/H_2O$	47/3/50	5 1 / 2 5	2 3 / 1 7	5 2 / 2 3	[5 1 / 1 2]	3 1 / 3 3
	50/10/40	5 1 / 2 5	2 4 / 1 8	4 1 / 2 3	6 5 / 1 2	3 1 / 3 2
	41/19/40	5 1 / 2 4	2 3 / 1 7	3 1 / 2 3	[6 4 / 0 2]	4 3 / 4 5
$1\text{-}BuOH/MeOH/H_2O$	48/2/50	5 1 / 4 4	5 4 / 6 10	[4 0 / 4 2]	[5 0 / 1 0]	3 1 / 4 2
	43/7/50	5 1 / 4 5	6 3 / 7 9	[4 0 / 3 1]	3 3 / 2 3	5 1 / 4 3
	38/12/50	5 2 / 5 5	6 3 / 8 10	5 3 / 3 3	4 1 / 2 2	4 2 / 4 3

[a] solvents used; 1-BuOH = 1-butanol, $CHCl_3$ = chloroform, EtOAc = ethyl acetate, MeOH = methanol, H_2O = water

[b] To simulate the orientation on the TLC plate, the scores are presented as follows.

	upper phase	lower phase
No. of spot with Rf>0.5	A	C
No. of spot with Rf<0.5	B	D

[c] Samples are the most polar fractions from; SS = *Senna siamea*, NT = *Narcissus* 'Sir Winston Churchill', EG = *Eucalyptus globulus*, VT = *Vitex trifolia*, OS = *Orthosiphon*

Figure 2. The evaluation of some solvent systems for the separation of five extracts shown by the number of the spots detected from TLC using a solvent system, ethyl acetate/formic acid/acetic acid/water 100:11:11:27 (v/v/v/v). The samples were dissolved in equal amounts of the two phases of the solvent systems tested as described in Experimental. The results showing that the systems are not suitable for CPC solvent systems are presented in dotted rectangles.

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a slight increase in performance when methanol was increased, therefore a two-phase system 43:22:35 (v/v/v) which showed the highest possible ratio of methanol was finally chosen.

Evaluation of CPC-2

The most polar CPC-1 fractions from five extracts were fractionated by CPC-2. The distribution of components among the subfractions was analyzed by means of TLC and the separation efficiency was evaluated by the method previously described.[3] For all extracts, satisfactory separation was obtained. The bioactivities of the fractions obtained from CPC-2 were determined and found to be spread over some fractions which probably need only one or two more separation steps to purify the active compounds (Table 3).

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Table 3

The Evaluation of the Separation of the Chemical Components from the Most Polar Fractions of the Five Extracts After Fractionation by CPC-2 and the Distribution of Bioactivity Among Subfractions*

Plant Extracts in CPC-2	Evaluation Score (1)	Evaluation Score (2)	Active Subfractions
Eucalyptus globulus	++	++	5-8[a]
Narcissus 'Sir Winston Churchill'	++	++	19, 22 - 25[b]
Orthosiphon stamineus	+	++	5 - 9[a]
Senna siamea	+	++	-[c]
Vitex trifolia	+	++	6[a]

* The scores vary from - to +++ determined by the TLC evaluation method previously described using the following TLC solvent systems: (**1**) chloroform/methanol 9:1 (v/v) and (**2**) ethyl acetate/formic acid/acetic acid/water 100:11:1:27 (v/v/v/v). [a] Fractions that could inhibit the spasmodic effect of 5 μg/mL in anti-asthmatic assay. [b] Fractions that show less than 20% of AChE activity at a concentration of 0.1 mg/mL (in the microplate assay for AChE inhibitory activity). [c] Not active in the radioligand binding assays or th AChE assay.

CONCLUSIONS

A separation scheme for general use in finding active compounds from plant alcoholic extracts was developed. After the first separation step by CPC-1 using the two-phase system heptane/ethyl acetate/methanol/water 6:1:6:1 (v/v/v/v), the fractions are screened for bioactivity. In case that the activity is found in the most polar fraction, the fraction will be separated by CPC-2 using the two-phase system ethyl acetate/methanol/water 43:22:35, v/v/v. In this way, common known active compounds could be rapidly identified and the unknown active compounds could be focussed on. Further purification may require only one or two more steps.

FUTURE PERSPECTIVE

In order to further develop the prefractionation procedure by CPC for industrial application, some points have to be considered. In this study, the bioactivities for the bioassay used were found only in the most polar fraction from the first CPC run. However, it is possible that less polar fractions will show affinity on other assays. For example, some steroids or triterpenoids which could have pharmaceutical importance, are normally present in nonpolar fractions. Therefore, different second CPC systems for each range of fractions from the first CPC run should be probably developed. This can be done by the method described in this article. The possibility for the automation of the separation procedure and for coupling this to high throughput screening is to be studied.

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