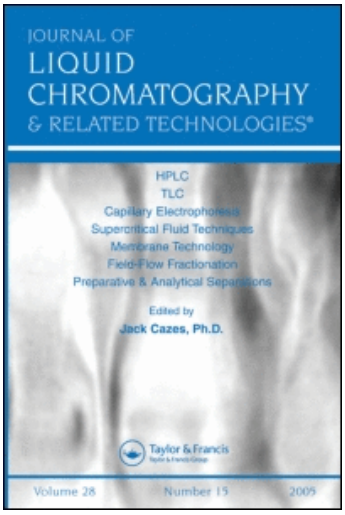


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# CCC in the Phytochemical Analysis of Anti-Tuberculosis Ethnobotanicals

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**Abstract:** Current paradigmatic approaches towards the identification of bioactive principles are built on a cyclic process known as bioassay-guided fractionation (BAGF). In our efforts to evaluate antimycobacterial leads from the ethnobotanical fundus of Alaska (*Oplopanax horridus*) and the Central-South Asian International Conservation and Biodiversity Group (ICBG) project (*Litsea mollifolia, Dubanga*

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*grandiflora*), a strategy involving countercurrent chromatography (CCC) of crude extracts improves the significance and prioritization power of early BAGF steps. One major factor is the high individual resolution of CCC having a small polarity window of separation, and leading to sharp individual resolution between constituents. The proposed strategy involves the CCC-based primary fractionation of extracts for two principal cases. Firstly, when working with unknown active constituents, the crude extract is divided into three main groups A ($K < 0.5$), B ($0.5 < K < 2$), and C ($K > 2$). As a result, the bioactivity pattern is restricted to 8 possibilities due to the nature of the separation, with a bioactive mass reduction of ca. 50–70%, depending on the extract and choice of solvent system. Secondly, when working with bioactivity targets, the polarity window can be specifically chosen around the lead constituent, resulting in significant enrichment of activity of 90% (w/w) or better.

**Keywords:** HSCCC, Ethnobotanicals, Bioactivity, Bioassay-directed fractionation, Bioassay-guided fractionation, Natural products, Drug discovery



## INTRODUCTION

It is estimated that one-third of the world's population tests positive for the presence of the tubercule bacillus, as each year an additional 8.8 million are infected.[1] Globally, tuberculosis results in the deaths of nearly 1.9 million people annually, giving it the distinction of the having the highest human mortality of all microbial species.[1,2] The WHO estimates that if efforts do not change, by 2020, nearly 1 billion additional people will be newly infected with tuberculosis, 200 million people will become sick, and 35 million will die of the disease.[1] Additionally, with the HIV/AIDS pandemic, the tuberculosis crisis is only exacerbated as the susceptible population dramatically increases.[3] Two very important elements have led to problems in the management of tuberculosis: (i) its endemism, primarily in countries of the developing world, and (ii) recent trends in drug-resistance.[1,4–7] It has been discussed that natural products have advantages as a source for drug leads due to their biological and structural diversity.[8] In addition, a number of compounds have been isolated from natural resources as anti-mycobacterials.[9] For these reasons, the successful identification of a plant or plant-derived compound that is effective against *Mycobacterium tuberculosis*, the causative agent of tuberculosis, would ameliorate global TB management efforts.

Common strategies to identify biologically active constituents of plants, involve chromatographic fractionation steps paired with subsequent biological testing against pathogens, deemed bioassay-guided fractionation (BAGF). While the most common methods of chromatography used with crude plant extracts involve adsorption chromatography, countercurrent chromatography (CCC), a partition chromatography method, is a powerful tool in the preliminary stages of crude extract fractionation.[10] With the ability to attain high resolution, modern CCC methods, such as high-speed countercurrent

chromatography (HSCCC), reduce the difficulties involved in natural product drug discovery, i.e. expensive and time-consuming steps to isolate active constituents. Due to the lack of a solid stationary phase, CCC has no irreversible absorption, therefore, providing loss-less fractionation. In crude plant fractionation this is particularly valuable, because it eliminates the chances of "losing" the activity during fractionation.[11] As a result, any loss of activity between countercurrent chromatographic steps can be confidently assigned to synergy between constituents. A specific method for utilizing CCC to concentrate crude plant extracts for biologically active constituents was designed with modifications for use in two distinct situations: (i) when the plant phytochemistry is completely unknown, and (ii) when an active constituent is suspected and can be targeted, either directly or through analogous compounds, which are on hand or commercially available. In order to assess the method in regards to both of the above scenarios, we report on the integration of CCC technology into the early BAGF process for two ongoing projects in our laboratory.



## EXPERIMENTAL

### Plant Collection and Extraction

*Litsea mollifolia, Dubanga grandiflora* were collected as part of the International Conservation and Biodiversity Group (ICBG) Associate Program-3 project for phytochemical analysis and biological screening. Supplemental voucher specimens are located in the John G. Searle Herbarium at the Field Museum in Chicago, IL, and the herbarium of the Institute of Ecology and Biology Research in Hanoi, Vietnam. Inner stem bark of *Oplopanax horridus* (Araliaeceae) from Alaska, was harvested from wild cultures by David C. Smith, Alaska Green Gold, Anchorage, in 2002. Voucher specimens are deposited in the company's herbarium.

Samples of *L. mollifolia* root (2.4 kg) and *D. grandiflora* stem bark (4.5 kg) were dried, ground and extracted with dichloromethane to yield 12.75 and 10.12 g of extract, respectively. In order to obtain full range of soluble compounds as part of the extracts, 157 g of powdered inner stem bark of *O. horridus* was exhaustively extracted with an Ultra Turrax® homogenizer, using three solvents of increasing polarity (dichloromethane, methanol, and 50% methanol in water) to yield 47 g of total extract.

### Solvent System Selection

Since there was no previous phytochemical work done on the plants used in the ICBG project, the ternary solvent system of $CHCl_3$:MeOH:$H_2O$ (5:6:4) was chosen based on its success in the literature for separating several known natural products that range in polarity.[12] For the Alaskan plant

project, the quaternary solvent system of petroleum ether:EtOAc:MeOH:$H_2O$ (7:3:6:4) was chosen. This decision was based on the K value of falcarindiol, a polyyne previously isolated from *O. horridus* with reported anti-TB activity in an agar-diffusion assay.[13] All organic solvents were HPLC grade from Fisher Scientific (Pittsburgh, PA, USA). Water was deionized to 18.2 $M\Omega \cdot cm$ at 25°C through the MilliQ Synthetic A10, Millipore Water (Bedfore, MA) system using a Quantum Ultrapure Cartridge® and fed through a double cartridge ion exchange system (Culligan, Northbrook, IL).

## High-Speed Countercurrent Chromatography Fractionation

Preliminary fractionation was conducted on a CCC-1000 J-type three-coiled planetary motion HSCCC (Pharma-Tech Research Corp., Baltimore, MD, USA), which has rotation radius (R) of 7.5 cm, using 3 × 108 mL PTFE Teflon coils with an inner diameter (i.d.) of 1.6 mm, an outer diameter (o.d.) of 2.7 mm, and beta values from 0.47 to 0.73 for all coils. Additionally, the follow up fractionation of *O. horridus* utilized a 3 × 283 mL PTFE Teflon coil (i.d. 2.6 mm, o.d. 4.1 mm) and beta values from 0.47 to 0.73. The HSCCC system was equipped with a Lab-Alliance Series III digital single-piston solvent pump, a Shimadzu SPD-10A VP UV-vis detector with preparative flow cell, a Cole-Parmer modular paperless recorder model 80807-00, and a Pharmacia Biotech RediFrac 95-tube fraction collector. On successive runs, samples of *D. grandiflora*, (389 mg) and *L. mollifolia* (296 mg) were dissolved in a mixture of 15 mL of lower organic phase and 10 mL of upper aqueous phase, and injected after equilibrating the HSCCC at 1000 rpm. The flow rate was 1.5 mL/min with upper phase as the aqueous mobile phase (reverse phase, tail in head out). The eluent was collected at 4 min intervals until K reached 2.1 (6.5 hours after injection). The stationary phase fraction ($S_F$) was calculated to be 0.75 and 0.70 for the *Dubanga* and *Litsea* runs, respectively. UV was recorded at 254 nm and 280 nm.

Two parallel runs were conducted for the Alaska project. For preliminary fractionation, an aliquot (662 mg) of *O. horridus* extract was dissolved in 5 mL of a 1:1 mixture of upper organic and lower aqueous phase. The HSCCC instrument with the 3 × 108 mL coil set was equilibrated with upper organic phase as the mobile phase (normal phase, tail in head out) and achieved a $S_F$ of 0.54 at a flow rate of 1.5 mL/min, rotating at 1000 rpm. The eluent was collected at 4 min intervals until K reached 3.2 (7 hours after injection). For follow up fractionation, a 1.2 g aliquot of the *O. horridus* extract was dissolved in 10 mL of a 1:1 mixture of upper and lower phase. The HSCCC with the 3 × 283 mL coil set was equilibrated in the same method and achieved a $S_F$ of 0.71 at a flow rate of 3 mL/min and a rotation speed of 1000 rpm. The eluent was collected at 3 min intervals until K reached 3.2 (12 hours since injection). Fractionations were monitored by UV absorption at 254 nm for both *Oplopanax* separations.

All HSCCC fractions were analyzed by thin layer chromatography (TLC). Alugram Silica G/$UV_{254}$ 10 × 20 cm plates with a thickness of 0.20 mm (Macherey-Nagel, Germany) were horizontally spotted with each fraction of eluent. While all samples were detected with a nonspecific *p*-anisaldehyde dipping reagent (96% glacial acetic acid, 2% $H_2SO_4$, 2% *p*-anisaldehyde), solvent systems used for TLC development were $CHCl_3$:MeOH (9:1, v/v) for the ICBG plants and Hexane:EtOAc (8:2, v/v) for *O. horridus.* Based on the TLC chromatograms and the UV detections from the HSCCC runs, each ICBG separation was combined to 12 fractions. The *Oplopanax* separations were combined to 12 fractions, and 64 fractions for the follow-up, larger scale separation. The 12 fraction and 64 fraction separations will be called low- and high-resolution fractionations, respectively, for the following discussion.



## Anti-Tubercular Assay

The anti-tubercular assay utilized was a Microplate Alamar Blue Assay (MABA) using virulent strains of *M. tuberculosis* H37R*v* and ERDMAN strains. This assay was chosen based on the advantages of small sample requirements, low cost, and high throughput capabilities, and has been validated against the clinical standard in susceptibility testing (BACTEC system).[14,15] Due to the number of fractions tested and the high minimum inhibitory concentrations (MIC) of crude fractions, percent inhibition at various concentrations were determined against drug-sensitive *M. tuberculosis* H37R*v* (ATCC 27294) and *M. tuberculosis* ERDMAN (ATCC 35801). Rifampin was used as a positive control.

## RESULTS AND DISCUSSION

### Significance of CCC in BAGF of Crude Extracts

In pharmacognostic research involving drug discovery and determination of active principles, one key concern is the ability to process and test plant material in an efficient manner. Using HSCCC, we have been able to produce effective preliminary fractionations of crude plant material to accelerate the bioassay-guided fractionation process. Since the MABA assay for testing percentage growth inhibition of *M. tuberculosis* is not an enzyme based or receptor binding *in vitro* assay, there is less of a concern regarding the elimination of compounds producing "false-positive" test results such as tannins, fatty acids or polyphenols.[12] Consequently, there is no need for the *a priori* elimination of those fractions that contain these compounds, since there is full legitimacy for a potential antitubercular compound to be present in the same polarity range.

Since any CCC separation for one given solvent system is focused on a relatively small polarity window, it is not only a powerful method for isolating a single target compound from a crude mixture, but also allows to precisely divide the analyzed sample into fractions. Thus, as shown in Figure 1, CCC can divide even a crude mixture into three main fractions based on the partition coefficient ($K_p$) in a highly reproducible manner. While separation utilizing silica gel-based adsorption chromatography has fairly consistently mediocre resolution over a wide (gradient) polarity range of the phases involved, the separation using countercurrent techniques maximizes resolution in a small segment of the total polarity range of the sample. Compounds outside of this segment elute with lower or no resolution, while compounds within the targeted polarity range (fraction B) are resolved more effectively than in separations using a solid stationary phase. Depending on the solvent system selection, this window of high-resolution can be shifted in either direction, i.e., more or less polar (Fig. 1). An important characteristic of CCC is that, assuming a solvent system of intermediate polarity with respect to a crude extract sample is used, fraction groups A and C can be predicted to contain the majority of the mass of the sample. As a result, an excellent preliminary fractionation is provided within the B range, where fractions can be combined based on TLC, UV, or even bioassay monitoring results. Using the strategy in the presented work, HSCCC runs of crude extracts typically resulted in an average of 12 fractions (A, $B_{1-10}$, C) that were submitted for bioassay, minimizing efforts with regards to both the bioassay and the recombination of fractions.

When paired with bioactivity testing, i.e., anti-TB activity, the proposed strategy results in 8 possible combinations of fractions that show activity:



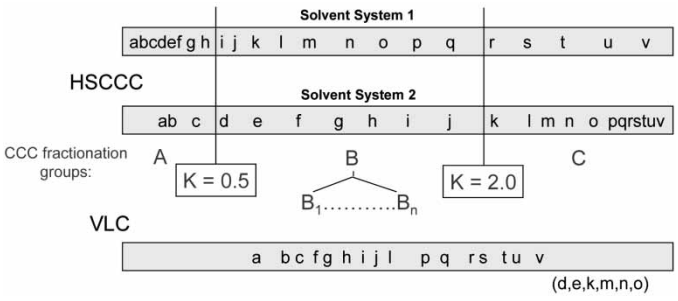


***Figure 1.*** HSCCC separation versus Vacuum Liquid Chromatography in the fractionation of crude extracts. In contrast to adsorption techniques (such as VLC) where irreversible absorption and decomposition (indicated as parentheses) are more likely to occur, CCC allows lossless separation. CCC separations using different solvent systems target different windows of polarity (group B) to isolate active principles. Cutting points were determined based on the level of expected resolution between fractions at certain K values.

(1–3) single fractions A, B, or C are active, (4–6) paired fractions A and B, B and C, or A and C are active, (7) all of the fractions are active, or (8) no activity is found. An ideal result is for the activity to be isolated to the B fraction, where activity can be correlated to a minimal fraction of original biomass that has been separated with high resolution. In the cases where the activities are isolated to A *and* C (scenario 6), the separation should be attempted with a different solvent system, in particular when either A or C are small in weight. The strength of the CCC method is derived from the fact that a result of either A + B, B, or B + C fractions possessing activity translates to an approximate 50 percent reduction in biomass without any loss of active constituents. Also of significance is that, when bioassays are performed in a quantitative and statistically significant manner, CCC separations ultimately allow proof of synergy, if activity is not present in fractions following separation of an active crude extract. This highlights one of the main benefits of this method. In column chromatography, where a solid support is used, the researcher is at a loss to whether a resulting lack of bioactivity is from multiple compound synergy, irreversible absorption of an active compound to the solid support, or chemical modification of an active compound by the stationary phase. In HSCCC this is rarely the case since the chromatographic process only exposes the extract to solvents and inert materials (mostly glass, stainless steel and Teflon). However, while there are caveats such as spontaneous rearrangements in solution (e.g. chalcone-flavanone isomerization), they certainly apply for any chromatography involving the use of classical solvents.[16]



## Two Scenarios for the Proposed Strategy

The strategy in using HSCCC for investigating active constituents from crude plant extracts is slightly modified based on whether or not the plants are known to contain active compounds. For this reason, data from two different projects have been included to demonstrate the versatility of this approach.

For the first project, plants devoid of previous phytochemical work were studied, through UIC's ICBG Project.[17] The goal of the project, as it relates to this paper, is to significantly concentrate the active constituents of plants collected from Vietnam in a single chromatographic step. In the second project, to identify anti-TB active principles from *O. horridus*, also known as devil's club, relevant phytochemical data had previously been published.[13] The plant has been used as traditional remedy for many ailments such as arthritis, digestive problems, and tuberculosis by indigenous tribes of the Northwest coast of North America.[18,19] It has been reported that *O. horridus* contains polyyne compounds that are active against mycobacteria in a agar disk assay,[13] yet no synergetic relation has been investigated. The goal of this project is to establish a more concise separation of the active principles and to find lead compound(s), as well as other (minor) active constituents.

## ICBG Project

In the ICBG project, two examples of plants initially fractionated with HSCCC have been included in the current study. In both of these examples there had been no previous phytochemical work done, and no published data on having anti-mycobacterial activity. When the crude extracts for *D. grandiflora* and *L. mollifolia* were tested against *M. tuberculosis* H37R*v*, they were shown to have minimum inhibitory concentrations (MIC) of 25 μg/mL and 12.5 μg/mL, respectively. Samples of each crude extract were fractionated using HSCCC and were monitored using a UV detector and the fraction were analyzed using TLC to show the predicted compound distribution, large fractions A and C, and several well separated B subfractions. When tested in the MABA assay, inhibitory activity was reported for fractions $B_9$ and C for *D. grandiflora* (Fig. 2), and fractions $B_2$, $B_{5-6}$, and C for *L. mollifolia.* This data concluded that more then one active compound was present in each of the plants and the compounds were not working synergistically. Biomass and complexity on TLC were used as factors to analyze the effectiveness of the separation, based on reducing biomass and concentrating active constituents. In the separation of *D. grandiflora*, fraction $B_9$ accounted for 2% of the total sample, and showed very few spots on TLC. The C fraction accounted for 60% sample biomass and showed



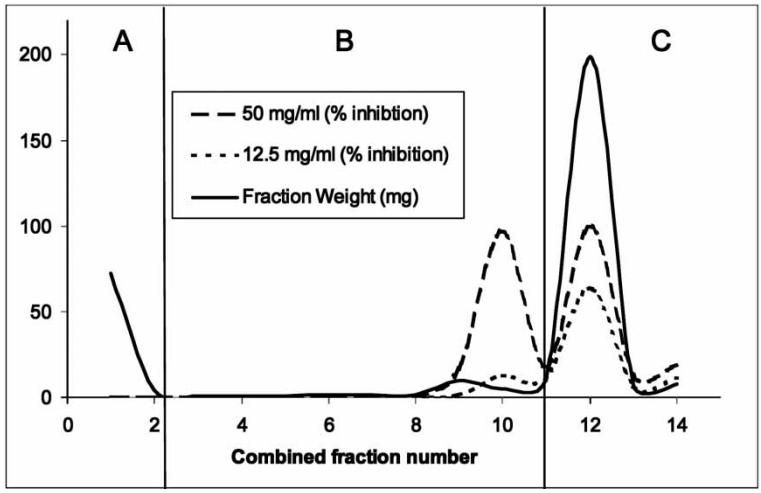


***Figure 2.*** Bioactivity versus fraction weight for *D. grandiflora* HSCCC preliminary fractionation. Abscissa: *In vitro* anti-TB activity (MABA, *M. tuberculosis*, H37R*v*) and weight in milligrams. Bioactivity was isolated to the $B_9$ and C fractions when combined based on the primary fractionation methods described above. This represents a significant biomass reduction in active fractions, with fractions $B_9$ and C accounting for 2% and 60% of the injected sample weight, respectively.

numerous compounds on TLC. This was considered a successful preliminary fractionation, since one active, minor constituent was nearly isolated in a single step and the remainder of the activity had been reduced to just over half of the original biomass. Additionally, follow up of the active C fraction is expedited with the knowledge of the $K_p$ within the solvent system, easing the solvent system selection process for a subsequent HSCCC experiment. Accurate solvent system selection will put the C fraction range of the preliminary fractionation into the 'B' range of polarity of the subsequent fractionation to isolate additional active-TB compounds. The separation was also successful for the extract of *L. mollifolia.* Active fraction $B_2$ accounted for only 3% of the samples biomass, and indicated a single spot on TLC. While this data followed the current proposed strategy, data from the other two remaining active fractions were seemingly exchanged. Fraction C, usually large in biomass, only accounts for 13% of the original biomass, whereas fraction $B_{5,6}$ accounts for 49% of the biomass of the injected sample. Both of these active fractions can be easily followed up by HSCCC with a modified solvent system for active compound purification.



## Alaska Project

The total extract of the Alaskan ethnobotanical *O. horridus*, which has been reported to produce anti-mycobacterial polyyne compounds, was shown to improve activity by up to 8-fold after a single HSCCC separation. A crude extract of *O. horridus* exhibited an MIC of 127 μg/mL against *M. tuberculosis* ERDMAN, versus preliminary CCC fractions that showed 15.6 μg/mL (B2) and 21.2 μg/mL (C1). Fractions B1 ($K = 0.50–0.81$) and C1 ($K = 2.00–3.12$) accounted for only 4 and 0.8% of the total sample weight, respectively. Again, the total sum of B fractions accounted for only 8% of the crude extract. While [HS]CCC is a high-resolution chromatography, the complexity of natural extracts is exceedingly high. Accordingly, TLC monitoring of fractions exhibited the persistence of natural complexity in those fractions. Approximately 60 compounds were shown to be present in the B range alone, while more can be expected to be unresolved or too low in (relative) concentration for TLC detection. Fraction B1 was shown to contain falcarindiol as a major component by TLC comparison with authentic sample, along with about 7 minor unidentified compounds.

The power of CCC can best be demonstrated by comparing low- and high-resolution biochromatograms of the *O. horridus* example. Biochromatograms are chromatograms resulting from pure biological detection, in this case detection of the anti-TB activity. In order to obtain a high-resolution biochromatogram, a larger scale follow-up fractionation was performed (See Experimental). In contrast to the previous low-resolution case, where fractions were combined based on UV and TLC detection, the high CCC resolution was composed by increasing the number of (combined) fractions

and by testing all of them in the bioassay. As a result, a high-resolution biochromatogram with a baseline separation of at least 10 different active, yet still combined fractions with 20 to 96% inhibition at 50 μg/mL concentration was achieved (Fig. 3). Most understandably, the high-resolution biochromatogram demonstrated that there are multiple active principles, rather than only two as would have been the conclusion from the low-resolution biochromatogram. Moreover, the high-resolution biochromatogram provided evidence for the presence of synergy between active principles, since subfractions exhibited reduced bioactivity when semi-quantitatively compared with their precursors and the crude extract. The most potent fraction had an MIC of 47 μg/mL, and was shown to contain falcarindiol by TLC comparison. One possible explanation for this observation is that active compounds co-elute with their enhancers as well as other non-active compounds in the low-resolution fractions. While the active compounds were separated from their enhancers in the high-resolution chromatogram, they exhibited lower activity due to the overwhelming presence of non-active compounds in the same fraction. Their singular activity may eventually recover as the active compounds are further purified. A fully quantitative evaluation of the observed synergy, based on conclusions drawn from the comparative biochromatograms, is an ongoing effort in our laboratory.



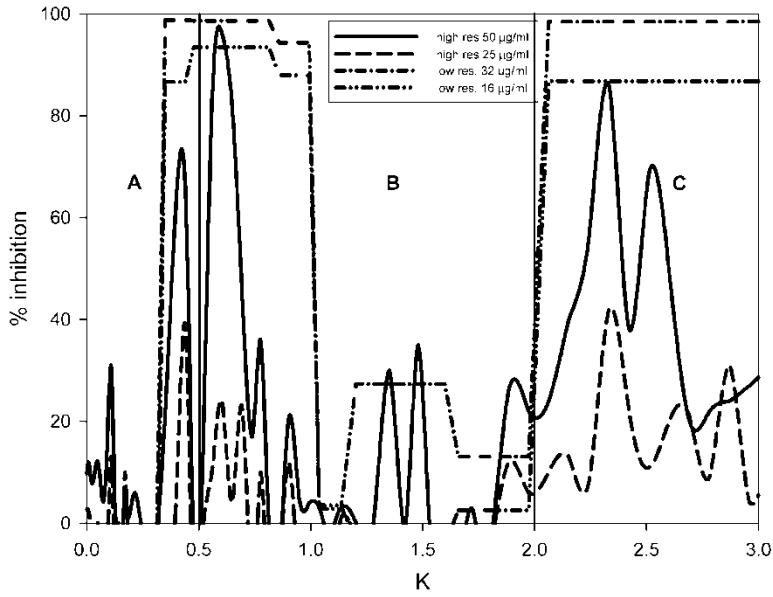


***Figure 3.*** High- versus low-resolution biochromatograms of *O. horridus.* Abscissa: *In vitro* anti-TB activity (MABA, *M. tuberculosis*, ERDMAN). What appears to be one principle in low-resolution, is shown to posses several different active principles when compared to the high-resolution biochromatogram. Note the increased activity in the low-resolution biochromatogram indicating synergism between active principles.

## CONCLUSIONS

When used for the fractionation of bioactive crude extracts, the most notable characteristics of countercurrent techniques are the significant reduction in biomass, concentration of active compounds in small fractions, and the enrichment of minor active constituents from a crude plant extract in a single chromatographic step. Other benefits, as shown from both the ICBG and the Alaska project data, include the conclusive evidence of a relatively large number of distinct sources of anti-TB bioactive principles in each plant, and inferred ease of follow-up experiments by simple adjustments of the solvent system being used. Additionally, in the Alaska project, the presence of synergistic effects became evident from the biochromatograms of two parallel separations recorded at different levels of resolution. The examples demonstrated that selecting a solvent system that maximizes the target section of the polarity scheme (the 'B' range) is essential in carrying out effective separations. In the case of the ICBG project, a general solvent system taken from the literature proved appropriate for plants with no phytochemical background. In the Alaska project, a solvent system guided by a lead compound achieved a biomass reduction to less than 5% and activity enhancement by up to 8-fold.



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