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Evaluation of Antibacterial Potential of *Quercus baloot* Using a Rapid Bioassay-Guided Approach

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Abstract: In the pursuit of novel drugs, natural products, especially those from herbal medicines are being investigated in more detail. Plant extracts are complex samples and whilst screening of crude samples is of some use, reliable screening data can only be obtained from purified material. This study describes the novel application of automated preparative-HPLC combined with a rapid off-line bacterial bioassay, using the tetrazolium salt, XTT reaction as an indicator of bacterial metabolism. This approach facilitated the identification of bioactive fractions from *Quercus baloot* that were active against *Staphylococcus aureus* and *Escherichia coli*. This is the first report of bioactivity associated with *Q. baloot*.

Key words: Preparative HPLC · XTT assay · Medicinal plants · Antibacterial · *Quercus baloot*

INTRODUCTION

The global occurrence of antibiotic resistant pathogens and the lack of antibiotic drug discovery programs in major pharmaceutical companies have led many researchers to explore new and revisit old sources of bioactive chemicals in order to obtain potential drugs and drug scaffolds [1]. These approaches include exploiting microbes from extreme environmental niches and manipulation of genes encoding secondary metabolite biosynthesis [2, 3]. Another area receiving attention is the investigation of the bioactive compounds involved in the many traditional herbal medicines used throughout developing areas of the world. Since a large number of the worlds drugs come from plants, there is no doubt that they are still a potential source of new drugs [4].

An evergreen shrub *Quercus baloot* Griff., known locally as Tor banj and it belongs to plant family Fagaceae It is commonly used for medicinal purposes in Pakistan. Whilst crude extracts of several species of *Quercus* have been shown to demonstrate antibacterial activity, there is little or no information on pharmacological and phytochemical properties of *Q. baloot*.

Isolation and characterization of bioactive natural products has been historically challenging and no doubt contributed to the decision of major pharmaceutical companies to develop high throughput technology for combinatorial strategies to deliver future drug candidates. To date, this expensive strategy has not delivered the drugs and pharmaceutical companies are moth-balling their compound factories [1]. However, a slow-resurgence in natural products is benefiting from many of the technological developments of the combinatorial age. As with combinatorial Libraries, preparative HPLC has been shown to be an essential tool for producing high quality natural products Libraries and combined with robust quality control ensures that screening results are reliable [5, 6]. In general, use of preparative HPLC for purification of bioactive natural products has been limited and generally manual systems used, with purification usually following a positive bioassay from a crude extract.

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The usual approach to measure antimicrobial activity is disk/well diffusion or growth assays, all of which are reliable but labour intensive and not ideal for large numbers of samples. Many mammalian cell viability assays use one of several tetrazolium salts, most commonly MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in a microplate format. Reduction of the tetrazolium salt by metabolically active cells results in the production of a coloured formazan product which can easily be analysed by a plate reader or digital scanner providing rapid data on the metabolic activity of the cell [5]. More recently XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) has been used as the reduced formazan product is water soluble and does not need the additional solubilisation step required in the MTT assay [7]. The XTT assay has been used to determine antimicrobial activity of simple plant extracts [8, 9] but has not been fully exploited. This manuscript describes the combination of automated preparative HPLC with the rapid XTT bioassay for the rapid identification of antibacterial fractions and derived sub fractions from *Q. baloot*.

MATERIALS AND METHODS

Chemicals: General purpose solvents, n-hexane, dicholoromenthane, ethyl acetate and n-butanol were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile and methanol were obtained from Rathburn (Walkerburn, Scotland). Trifluoroacetic acid (TFA) and dimethyl sulfoxide (DMSO) were supplied by Fisher (Loughborough, UK). Water was purified using a Milli-Q system (Millipore, Watford, UK). XTT sodium salt, Menadione sodium bisulfite (2-Methyl-1,4-naphthoquinone sodium bisulfite), ciprofloxacin and clarithromycin were obtained from Sigma (Poole, UK). Nutrient broth and agar, Mueller-Hinton agar and phosphate buffered saline (PBS) were obtained from Oxoid (Fisher, Loughborough, UK).

Bacterial Strains: *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 25922) from the American Type Culture Collection (ATCC: Manassas, USA) were used as the preliminary screening bacteria for this study. *E. coli* (NCIMB 8797) and *S. aureus* (NCIMB 6571) from the National Collection of Industrial, Marine and Food Bacteria (NCIMB; Aberdeen, UK) were used for the XTT bioassay.

Plant Material: The leaves of the *Quercus baloot* plant were collected from Swat (Latitude 35° 0' 0" and Longitude 72° 30' 0"), NWFP, Pakistan, in October 2007. A specimen was matched for confirmation of identity with the reference voucher number 379, preserved in the Herbarium of Pakistan.

Extraction and Fractionation: Shade dried leaves of *Q. baloot* (15 kg) were ground and extracted with methanol (80% *v/v*) and dried under vacuum at a temperature not exceeding 40°C, giving 3.2 l of crude extract. Crude aqueous fractions of both plants were sequentially partitioned with hexane (3 x 1 l), dichloromethane (3 x 1 l), ethyl acetate (3 x 1l) and n-butanol (3 x 1 l) and combined extractions were dried by rotary evaporation [10].

HPLC: The HPLC system consisted of a Waters Alliance 2695 with a 2996 photodiode array detector (PDA) and a ZQ 2000 mass spectrometer (Elstree UK). The separation was performed on a Sunfire C18 column (Length 150 mm *x* 2.1 mm I.D., particle size 5 µm) which was maintained at 40°C. Mobile phase was Milli-Q water (A) and acetonitrile (B) both containing 0.05% TFA. Samples (10 µl) were separated using a gradient increasing from 5% to 100% B over 30 minutes at a flow rate of 0.3 ml/min. Eluent was monitored from 210-400 nm with a resolution 1.2 nm and by positive ion electrospray (ESI+) in series, scanning from *m/z* 100 to 1600 with a scan time of 2 s and inter-scan delay of 0.1 s. Ion source parameters; sprayer voltage, 3.07 KV; cone voltage, 40 V; desolvation temperature, 300°C; and source temperature, 100°C. Instrument control, data acquisition and processing were achieved using MassLynx v4.1.

Preparative HPLC was performed using a Biotage Parallex Flex (Biotage, Cardiff, UK) where Flex V3 software was used for instrument control and data acquisition. Separations were performed on a Hyperprep HS C18 column (10 mm I.D. x 150 mm long; 8 µm particle size; Thermoscientific, UK) with a pre column (KR 100-13 C18-10 CP; Hichrom Ltd., Berkshire, UK). Mobile phase consisted of Milli-Q water (A) and methanol (B). The crude samples were separated using a gradient increasing from 10 to 100% B over 50 min at a flow rate of 10 ml/min. The concentration of crude fractions was 0.2 g/ml and each injection volume was 0.25 ml. Eluent was monitored at 220 nm and 254 nm and fractionation was volume based, collecting 9 mL fractions into deep well microtitre plates (24 x 10 ml; Whatman, Kent, UK).

Fractions from the generic separations that exhibited antibacterial activity were dried, weighed and re-dissolved in 0.5 ml methanol. These were further purified using polarity focused gradients based on the proportion of methanol in which they were eluted during the generic gradient (Table 2). Column and solvents were as described previously with an injection volume of 0.25 ml. Fraction collection was based on volume (9 ml fraction).

Preliminary Antibacterial Screening: Crude extracts (16.7 mg/ml) were re-suspended in methanol and sterilized using Millex, 33 mm filter (0.22 µm pore size; Millipore, Watford, UK). Aliquots (6 µl) were applied to sterile disks (6 mm diameter punched from Whatman No. 1 filters). Negative control disks were prepared by applying the same volume of methanol to each disk. Ciprofloxacin (8.4 mg/ml in 0.1N HCl) and clarithromycin (3.3 mg/ml in 0.1N HCl) were the positive controls for *E. coli* NCIMB 8797 and *S. aureus* NCIMB 6571 respectively. The bacteria were maintained on nutrient agar and standardized inocula were prepared by culturing the bacteria in nutrient broth. Inocula were calibrated to turbidity equivalent to 0.5 McFarland turbidity standard [11] using PBS. An aliquot (100 µl) of the standardized suspension was evenly spread on Mueller-Hinton agar and allowed to dry. Using sterile forceps the disks containing the crude extracts, negative control disks (methanol) and positive controls were placed on the seeded plates. The plates were kept in inverted position in refrigerator for 2 h to allow adequate diffusion of extracts. Zones of inhibition were measured after 24 h incubation at 37°C. All the tests were performed in triplicate.

XTT Bioassay: XTT solution (1 mg/ml) in PBS, was prepared and sterilized as described previously for the crude extracts. Cryotubes (Cole-Parmer, London, UK) containing 1 ml aliquots were kept at -80°C until required. Menadione (173µg/ml) in acetone was prepared prior to every assay. For each assay, XTT aliquots were thawed at room temperature and then one part menadione added per 12 parts XTT (1:12). From each fraction collected during prep-HPLC an aliquot (300 µl) was placed in glass vials (2 ml; Alltech, Carnforth, UK) and dried overnight in a laminar flow cabinet and re-suspended in DMSO/PBS (400 µl). Samples (100 µl) were transferred into the wells of 96-well flat bottom microtiter plates (Sterilin, Aberbargoed, UK).

E. coli NCIMB 8797 and *S. aureus* NCIMB 6571 were cultured on nutrient agar and single isolated colonies were transferred to flasks of nutrient broth (100 ml), incubated at 37°C and shaken at 150 rpm for 18 h. An aliquot (1 ml) of the overnight culture was used to inoculate a second flask of nutrient broth and incubated as described for 2 h to enable the bacteria to reach mid-log growth phase. After 2 h optical density (OD; 600 nm) was measured and cultures were diluted with sterile PBS in order to obtain an OD of 0.03 which had been determined to be approximately 5 x 10^5 CFU/ml in previous growth experiments. Inoculum (100 µl) of each test bacteria was added to each of the test well, into negative control (DMSO/PBS) and positive control (ciprofloxacin for *E. coli* and clarithromycin for *S. aureus*) wells. XTT/Menadione (18 µl) was added to each test well and plates were gently shaken. Absorbance at 490 nm was recorded using a BioTek microplate reader, (Fisher, Loughborough, UK) at the start of the test and after 24 hours of incubation at 37°C. All tests were performed in duplicate.

Percent increase in absorbance (X) was calculated using formula 1 where A_{tN} and A_{t0} represented mean absorbance values at specific time (24 h was standard) and zero time respectively. Percentage increase in negative control (N) was also calculated using formula 1. Percent reduction (R) in cell metabolism was calculated using formula 2 where X_N and T_N represented % increase in absorbance of test material and negative control respectively at time (N) which was standardized at 24 h.

$$X_{tN} = A_{tN} - A_{t0}/A_{t0} \text{ x } 100 \quad (1)$$

$$R = 100 - [(X_N/T_N]) \text{ x } 100] \quad (2)$$

Fractions were deemed to have an antimicrobial effect when they gave a mean (n = 2) reduction in the XTT colorimetric bioassay of ≥ 50% (threshold level for antimicrobial activity) and <50% value was termed to have little or no antimicrobial activity [9].

RESULTS AND DISCUSSION

Liquid-liquid fractionation of the crude aqueous extract of *Q. baloot* gave gummy n-hexane (20 g), highly viscous dichloromethane (140 g) and ethyl acetate (102 g), gummy *n*-butanol (75 g) and an aqueous fraction (135 g). Preliminary Antibacterial Screening: The dicholoromethane extract of *Q. baloot* demonstrated

Table 1: Antibacterial activity of crude solvent fractions determined by disk diffusion assay.

Fraction	*E. coli* (NCIMB 8797)	*S. aureus* (NCIMB 6571)
Hexane (H)	-	-
DCM (D)	+	+
Ethyl acetate (E)	-	-
Butanol (B)	-	-
Aqueous (A)	-	-

+ inhibition of bacterial growth (> 10 mm diameter)

- no inhibition of bacterial growth (< 10 mm diameter)

Single letter abbreviation for extracted fraction given in parenthesis

Table 2: Summary of bioactive fractions from extracts of *Q. baloot* separated by generic gradient preparative HPLC

	Antibacterial activity[a]			
Sample - fraction (mg dry weight)	*E. coli* (NCIMB 8797)	*S. aureus* (NCIMB 6571)	Percentage methanol	Focused gradient
Q. baloot – D[b]1 (0.5)	++	-	10	0-20 %
Q. baloot – D37 (1.6)	+	+	74	60-90 %
Q. baloot – D38 (0.8)	+	-	76	
Q. baloot – D39 (1.7)	+	+	78	
Q. baloot – D40 (1.2)	+	-	79	

a - Antibacterial activity as determined by XTT assay:

++ represents ≥ 75% reduction in bacterial activity,

+ represents 50-74% reduction in bacterial activity

- represents <50% reduction in bacterial activity

b - Fractions from crude dichloromethane extract (D).

Table 3: Summary of bioactive fractions from focused prep-HPLC runs of fractions from *Q. baloot*

		Antibacterial activity	
Sample - Primary Fraction	Sub-Fraction	*E. coli* (NCIMB 8797)	*S. aureus* (NCIMB 6571)
Q. baloot – D38	9	-	+
	13	+	-
	18	+	-
	19	+	-
Q. baloot – D39	15	+	++
	16	+	++
Q. baloot – D40	17	+	-
	18	+	-
	19	+	-

++ represents = 75% reduction in bacterial activity,

+ represents 50-74% reduction in bacterial activity

- represents <50% reduction in bacterial activity

activity against *E. coli* (NCIMB 8797) and *S. aureus* (NCIMB 6571) representing, to our knowledge, the first report of antibacterial activity from this species (Table 1). The aqueous extract of the plant were inactive against both bacteria. Whilst there is growing interest in the generation of Libraries of simplified extracts prior to screening [5, 6], our approach was to select those extracts which exhibited antibacterial activity and use preparative HPLC with the XTT assay for rapid screening of large numbers of fractions.

HPLC – XTT Bioassay: A generic gradient (10-100% water:methanol) was used to separate the four extracts which demonstrated antibacterial activity. The use of simple solvents of methanol and water along with a volume based, fractionation strategy was necessary as the active component(s) may not contain a chromophore. Five fractions from the dichloromethane extract of *Q. baloot* exhibited activity (Table 2). A polar fraction (D1) was highly active against *E. coli*, whereas the remaining fractions were moderately active against *S. aureus*.

Fig. 1 shows the data from the preparative HPLC with the XTT assay results, linking the active fractions to the corresponding peaks in the chromatogram, indicating the hydrophobic nature of the components in the fractions.

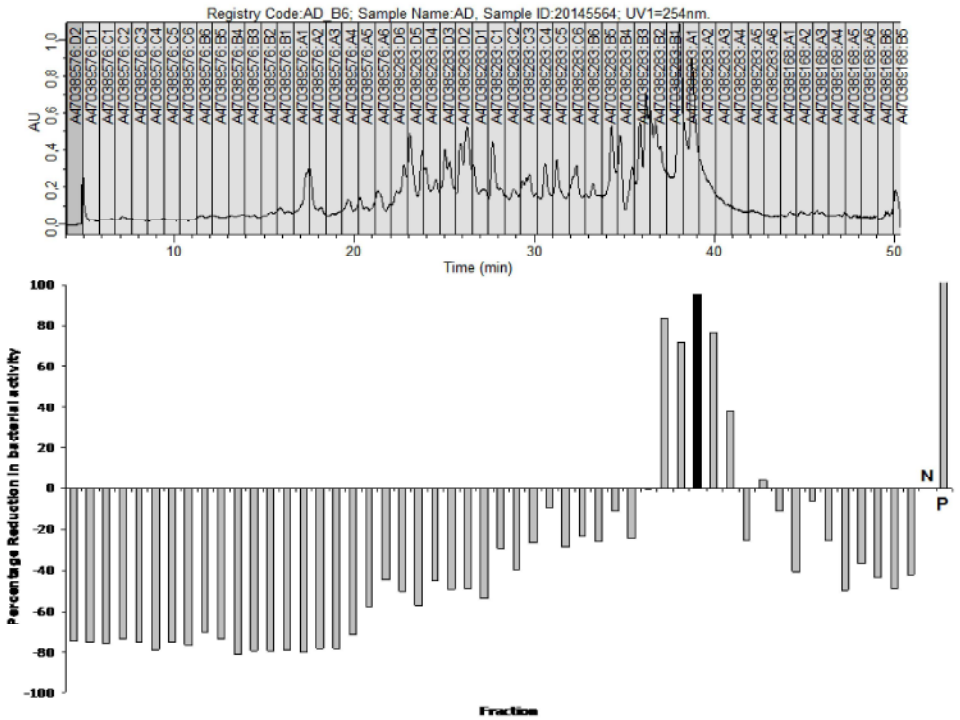

Fig. 1: Preparative separation of dichloromethane extract of *Q. baloot* using generic gradient and volume based fractionation (above) susceptabilities of *S. aureus* (NCIMB 6571) as determined by XTT bioassay (below) where N represented the negative control of DMSO/PBS and P the positive control of ciprofloxacin (n=2 ± SD). Fraction 39 (black) contained high purity, bioactive compound

Whilst fractions which resulted in a reduction of bacterial activity were clearly evident, the majority of fractions gave enhanced bacterial activity, most likely as due to additional carbon sources. Using the data available on the percentage of methanol eluting the bioactive fraction, shorter (30 min) focused gradients were selected for further purification of all the primary bioactive fractions as summarized for each fraction in Table 2.

A total five bioactive fractions from *Q. baloot* were further purified using focused gradients over 30 min. Each separation resulted in 35 fractions which were screened by the rapid XTT assay. The polar fraction (D1) and fraction D37 from *Q. baloot* gave no bioactive fractions when separated using the focused gradient and was investigated no further. Sub-fractions from D38 and D40 demonstrated only moderate activity towards *E. coli* (Table 3). However, the sub-fractions from separation of D39 demonstrated strong activity towards *S. aureus* and moderate activity towards *E. coli* (Table 3).

Fig. 3 shows the separation and fractionation of primary fraction D39 from *Q. baloot* using a focused gradient (60-90% methanol over 30 min) with the corresponding results from the XTT assay showing susceptibility to *S. aureus* compared to the positive control clarithromycin. Extracts from other *Quercus* species have been shown to have antibacterial potential [12, 13] but to our best knowledge this is the first report of antibacterial activities from *Q. baloot*.

Preliminary Characterisation of Bioactive Compounds: The combination of preparative HPLC with the XTT assay rapidly enabled the isolation of multiple bioactive fractions from the plant. In addition, analysis by LC-PDA-MS of bioactive fractions following each of purification enabled information on fraction purity and physicochemical properties to be obtained. The active component in the dichloromethane extract of *Q. baloot* found in D39 (Fig. 2) and sub-fraction 15 (Fig. 3) where the relative purity (PDA – 210 to 400 nm) achieved was 90 and 95% respectively (Fig. 3 (c). This compound had an *m/z* 847 amu and UV absorbance maxima at 267 and 315 nm. Whilst this information alone was not sufficient for full characterization of the compound, it provided essential information for monitoring purifications for obtaining larger amounts of compounds, eliminating the need for unnecessary bioassays.

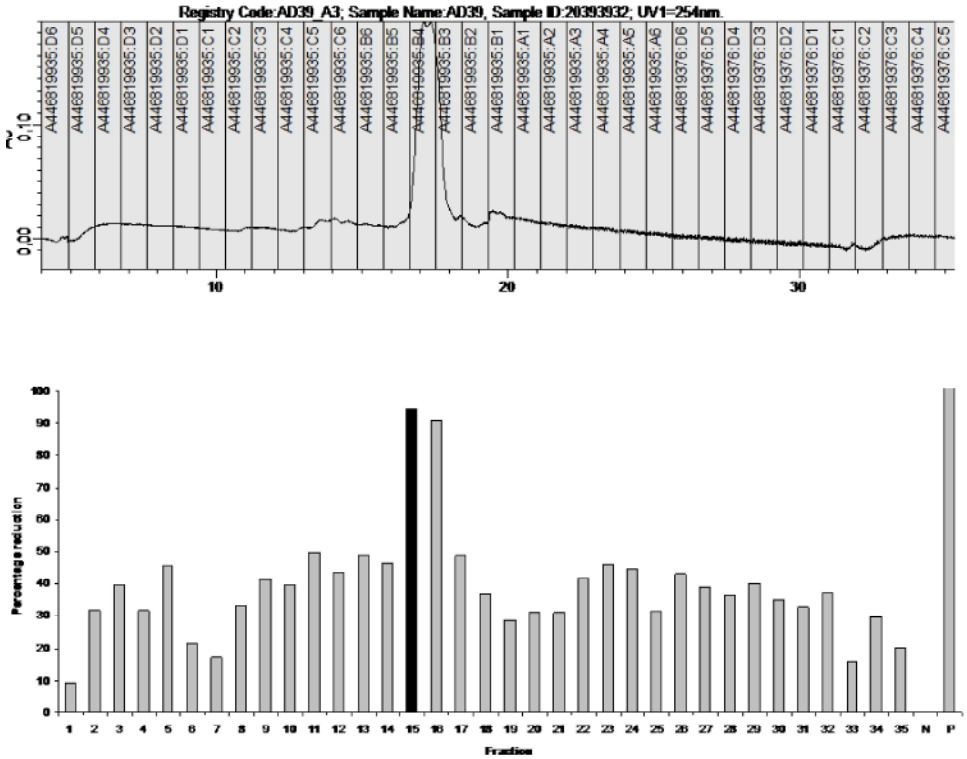

Fig. 2: Separation and fractionation of bioactive fraction 39 from dichloromethane extract of *Q. baloot* using polarity focused gradient (above) and corresponding susceptabilities of *S. aureus* (NCIMB 6571) as determined by XTT bioassay (below) where N represented the negative control of DMSO/PBS and P the positive control of ciprofloxacin (n=2 ± SD). Fraction 15 (black) contained high purity, bioactive compound

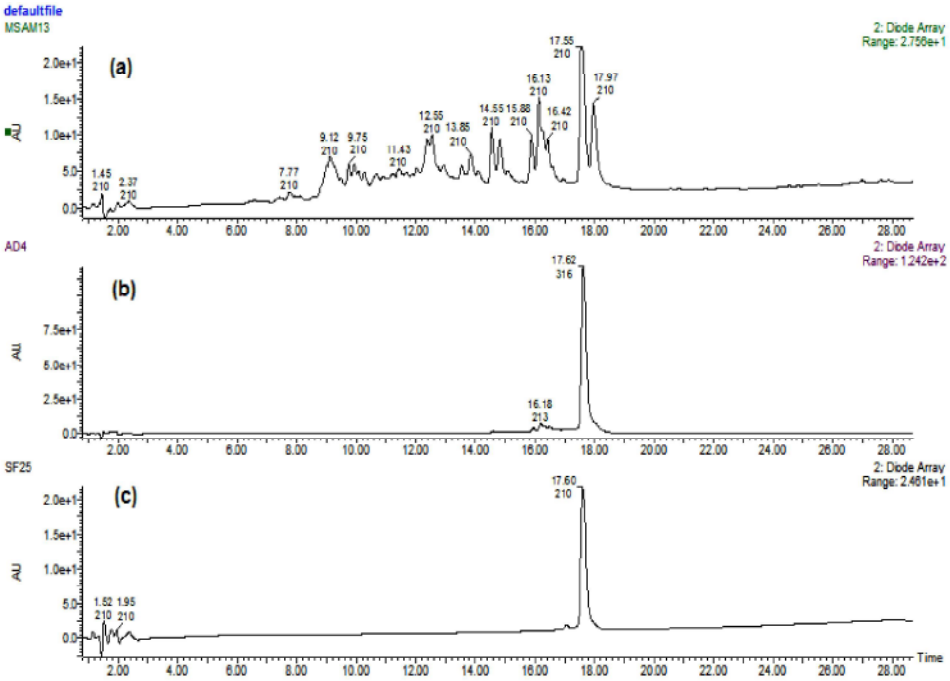

Fig. 3: (a) Separation of crude dichloromethane extract *Q. baloot* by reversed phase HPLC; (b) bioactive fraction 39 from generic preparative separation; and (c) bioactive fraction 16 from polarity focussed separation

CONCLUSIONS

The combination of automated semi-preparative HPLC with simple generic gradients followed by focused gradients combined with the XTT bioassay led to the rapid identification of sub-fractions bearing strong antibacterial activity. This approach resulted in detection of high purity bioactive compounds. Although two compounds were isolated during this study, there were clearly several bioactive compounds which require further investigation. Since some of these had no chromophore or did not ionize under experimental conditions used, the incorporation of a non-specific detector, such as evaporative light scattering, would be of great benefit [14].

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