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Abstract: Antioxidant potential of four methanol extracts from three selected plant species, namely Salvia nubicola (Lamiaceae), Acer oblongifolium (Aceraceae) and Hedera nepalensis (Araliaceae) was measured using assays in aqueous and lipid systems. Antioxidant activities were investigated in aqueous systems byusing DPPH radical-scavenging assay, ABTS radical-scavenging assay and DNA protection assay, while antioxidant activity in a lipid system was determined by using the thiobarbituric acid-reactive substances(TBARS) assay. Additionally, the Folin-Ciocalteu method was used to measure total phenolic content.Methanol extracts of leaves and flowers of S. nubicola showed the highest Trolox equivalent (TE) valuesin the case of the DPPH -- CORRECTION REQUIRED HERE -- 2; assay, 2484 -± 4.9 mmol TE/g extract, as well as total phenolic content,139 -± 0.2 mg gallic acid equivalents/g extract. Three fractions (A-€“C) of the methanol extract of S. nubicolaleaves and flowers were produced by semi-preparative HPLC. Fraction B was found to be the most activein the DPPH radical-scavenging assay and had the highest total phenol content. HPLC-DAD and LC-€“MSrevealed rosmarinic acid in S. nubicola extracts and chlorogenic acid and rutin in H. nepalensis extracts as the main phenolic antioxidants.

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Bioprospecting traditional Pakistani medicinal plants for potent antioxidants

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Abstract

Antioxidant potential of four methanol extracts from three selected plant species i.e *Salvia nubicola* (Lamiaceae), *Acer oblongifolium* (Aceraceae) and *Hedera nepalensis* (Araliaceae) was measured using assays in aqueous and lipid systems. Antioxidant activities were investigated in aqueous systems by using DPPH radical scavenging assay, ABTS radical scavenging assay and DNA protection assay, while antioxidant activity in a lipid system was determined by using the thiobarbituric acid reactive substances (TBARS) assay. Additionally, the Folin-Ciocalteu method was used to measure total phenolic content. Methanol extracts of leaves and flower of *S. nubicola* showed the highest Trolox equivalent (TE) values in the case of the DPPH assay, 2484.1 ± 4.9 mM TE/g extract, as well as total phenolic content, 138.8 ± 0.2 mg gallic acid equivalents/g extract. Three fractions (A, B, and C) of the methanol extract of *S. nubicola* leaves and flowers were produced by semi-preparative HPLC. Fraction B was found to be the most active in the DPPH radical scavenging assay and had the highest total phenol content. HPLC-DAD and LC-MS revealed rosmarinic acid in *S. nubicola* extracts and chlorogenic acid and rutin in *H. nepalensis* extracts as the main phenolic antioxidants.

Keywords: *Salvia nubicola* (Lamiaceae), *Acer oblongifolium* (Aceraceae), *Hedera nepalensis* (Araliaceae), antioxidant assays, Folin-Ciocalteau, bioactivity guided fractionation

1. Introduction

Antioxidants have widespread applications in medicine, cosmetics and food industries. Antioxidants from natural sources are gaining more acceptance due to emerging concerns about safety of synthetic preservatives. Although natural products have been the source of many modern pharmaceuticals, yet there is renewed interest in traditional medicine to provide new compounds with potent bioactivities, including much interest in antioxidant activity (Liu & Henkel, 2002). Further, while much research has focussed on traditional Chinese medicine, other cultures also have a long history of alternative medicine including those from Western Asia (Azaizeh, Saad, Cooper & Said, 2010).

As part of a screening study of traditional Pakistani medicinal plants (Inayatullah, Irum, Rehman, Chaudhary & Mirza, 2007), three plant species have been selected for further evaluation of their antioxidant activity. As no previous study describes antioxidant potential of selected medicinal plant species, the current study will provide useful information in this regard. Thus, the present study investigated methanol extracts of: *Salvia nubicola* leaves and flowers (SN-L&F) and *S. nubicola* stem (SN-S), family Lamiaceae; *Hedera nepalensis* leaves and stem (HN-L&S), family Araliaceae; and *Acer oblongifolium* leaves and stem (AO-L&S), family Aceraceae. A number of other *Salvia* species i.e. *S. virgata*, *S. staminea*, *S. verbenaca*, *S. officinalis*, *S. glutinosa*, *S. sclarea*, and *S. athiopis* are known for their antioxidant activity, which was attributed to varying quantities of rosmarinic acid, carnosic acid, carnasol, hydroxycinnamic acid derivatives (caffeic acid), benzoic acid derivatives, flavonoids (luteolin derivatives), and diterpenoids (Akkol, Goger, Kosar & Baser, 2008; Bandoniene, Murkovic, Pfannhauser, Venskutonis & Gruzdiene, 2002; Kosar, Dorman, Baser & Hiltunen, 2004; Tepe, 2008). *H. nepalensis* has some reported

anticancer, antidiabetic and cytotoxic activities (Gilani, Qureshi & Farooq, 2001; Inayatullah et al., 2007). *A. oblongifolium* has shown antitumor, cytotoxic and phytotoxic potential (Inayatullah et al., 2007). A number of Acer species are reported to have antioxidant activities including *A. albopurpurascens* (Jiang, Chang, Wen, Lin, Hsu & Lee, 2006; Lee, Jiang, Juan, Lin & Hou, 2006), *A. palmatum* (Kim et al., 2005), *A. nikoense*, *A. buerferianum* (Hou et al., 2003) and *A. saccharum* (van den Berg & Perkins, 2007). Their major antioxidant constituents have been identified as (+)-rhododendrol, (+)-catechin and vitexin.

The objective of the present study is identification and isolation of potent antioxidant compounds from the selected plant extracts. For this purpose, multidimensional antioxidant activity was determined in aqueous (DPPH radical scavenging assay, $ABTS^{\bullet+}$ scavenging assay and DNA protection assay) and lipid (thiobarbituric acid reactive substances, TBARS) systems as recommended (Frankel & Meyer, 2000; McDonald, Prenzler, Antolovich & Robards, 2001). This is the first report, as far as the authors are aware, of a systemic study to locate potent antioxidants from *S. nubicola*, *H. nepalensis*, and *A. oblongifolium*.

2. Materials and methods

2.1 Chemicals and reagents

Reagents used without further purification were Folin-Ciocalteu reagent, 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) and linoleic acid from Sigma-Aldrich (Sydney, Australia); 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and thiobarbituric acid from Fluka (Sydney, Australia); HPLC-grade methanol and *n*-hexane from Mallinckrodt (Paris, KY); anhydrous

acetonitrile from Unichrome (Sydney, Australia); glacial acetic acid, hydrochloric acid (32%), potassium persulfate and sodium carbonate from Univar (Sydney, Australia); sodium hydroxide and absolute ethanol from Biolab (Melbourne, Australia); and butylated hydroxy toluene (BHT) from ICN Biomedicals (Ohio, USA). Water used in all analytical work was purified by a Modulab Analytical water system(Continental Water Systems Corp., Melbourne, Australia). Phenolic standards used without further purification were gallic acid, caffeic acid and rutin from Sigma-Aldrich (Sydney, Australia).

2.2 Collection of plant material

Plant material was collected in July, 2005 from Northern areas (Swat and Kalam District, North-West Frontier Province, Pakistan) and dried at room temperature. The species and plant parts collected were leaves, flowers and stems from *Salvia nubicola*; leaves and stems from *Hedera nepalensis*; and leaves and stems from *Acer oblongifolium*. Dried material was crushed using a grinder and stored at −70 °C. Plant samples were identified at the Taxonomy Laboratory, Department of Plant Sciences, Quaid-i-Azam University, Islamabad by Professor Dr Mir Ajab Khan. Voucher specimens were deposited at the Taxonomy Laboratory.

2.3 Preparation of plant extracts

The four plant extracts (Table 1) were prepared by a maceration technique. Plant material was soaked in methanol for 7 days at room temperature, filtered and concentrated with a rotary evaporator at 40 °C under low pressure. The plant extracts were freeze-dried and stored at −20 °C.

2.4 Spectrophotometric scanning of hexane washes and extracts

Ten milligrams of the powdered plant extract were dissolved in 100 mL 80% aqueous methanol. Extracts were washed three times with 100 mL n-hexane to remove lipophylic pigments and lipids. Samples were filtered using GF/F (glass fibre filters) followed by 0.2 µm nylon syringe filters (Millipore, Sydney, Australia). Spectrophotometric measurements (200-800 nm) were performed on a Carry WinUV spectrophotometer (Varian, Australia). Spectra of methanol crude extracts (100 ppm) were obtained before and after hexane washing. Hexane washes were also scanned.

2.5 Determination of total phenolic content

Folin-Ciocalteu reagent was used for determination of total phenolic content as described earlier (Obied, Allen, Bedgood, Prenzler & Robards, 2005). Absorbance was measured at 760 nm. Data were expressed in terms of mg gallic acid equivalents (GAE)/g of extract (6 point regression curve, 0 – 250 mg/L, $R^2 = 0.987$) and/or as Trolox equivalents (TE)/g extract (6 point regression curve, 0.0 – 0.1 mM, $R^2 = 0.992$).

2.6 Antioxidant assays

2.6.1 DPPH radical scavenging activity

The DPPH assay was performed as described earlier (Obied et al., 2005). Absorbance was measured at 517 nm. Absorbance of 80% methanol was considered as blank while negative control (DPPH solution) was also run simultaneously. Trolox was used as a positive control. The percentage scavenging of DPPH radical was calculated according to the formula:

% scavenging = $100 \times (A_{control} - A_{sample})/A_{control}$

Results are expressed as mM Trolox equivalents (TE)/g extract based on an 8 point regression curve, 0.00 – 0.03 mM, $R^2 = 0.994$.

2.6.2 ABTS radical scavenging assay

The $ABTS^{\bullet+}$ scavenging assay was performed using a modified method of Paixao et al. (Paixao, Perestrelo, Marques & Camara, 2007). ABTS was dissolved in water to give a stock solution of 7mM. The ABTS radical cation was produced by reacting the stock solution with 2.45 mM (final concentration) potassium persulfate solution. This working solution was kept in dark at room temperature for 12 h prior to use. The working solution was diluted 50 fold with phosphate buffer (pH 8.04) and the absorbance was measured as 0.70 at 415 nm. Three µL, 15 µL and 30 µL of plant extracts were added to 3 mL of the ABTS radical cation solution in a cuvette (final concentration as 1 ppm, 5 ppm and 10 ppm respectively). Trolox was used as a positive control while the ABTS radical cation solution served as a negative control. The reduction in absorbance was measured at 415 nm. The percentage scavenging of the ABTS radical cation was calculated according to the formula:

% scavenging = $100 \times (A_{control} - A_{sample})/A_{control}$

Data are expressed in terms of mM TE/g extract based on a 5 point regression curve, 0.00 – 0.32 mM, $R^2 = 0.996$.

2.6.3 Thiobarbituric acid reactive substances (TBARS) Assay

TBARS for four plant extracts were determined by the method described by McDonald et al. (McDonald et al., 2001). Initial stock solutions of methanol extracts were prepared at concentrations of 10,000, 50,000 and 100,000 ppm in 100% methanol. 300 µL of $CuCl_2$ solution (0.05 mM) was added to each test tube followed by the addition of 50 µL of test solution and 100 µL of linoleic acid. The mixture was vortexed for five seconds and incubated at 37 °C in a shaking water bath for 20 hours. The reaction was stopped by the addition of BHT (20 µL, 10mM) and freshly prepared thiobarbituric acid (3ml, 0.67% in 0.1 M HCl). The reaction mixture was vortexed for five seconds and placed in a boiling water bath for 10 minutes. The tubes were allowed to cool and the pink aqueous layer was transferred to another test tube containing 2.5 mL of n-butanol. The mixture was vortexed for five seconds and allowed to settle. Absorbance of the pink layer was measured at 532 nm. *n*-Butanol served as blank while negative control (without any test substance) was run simultaneously. Trolox was used as a positive control. Percentage inhibition of TBARS was calculated as:

$$\% \text{ inhibition} = 100 \times (A_{control} - A_{sample})/A_{control}$$

Data are expressed in terms of mM TE/g extract based on a 6 point regression curve, 0 – 40 mM, $R^2 = 0.911$.

2.6.4 DNA protection assay

Pro-oxidant and antioxidant potential of plant extracts and fractions were measured by DNA protection assay as described earlier (Obied, Prenzler, Konczak, Rehman & Robards, 2009).

Plasmid DNA (pBR322, Fermentas) was diluted two fold with phosphate buffer (pH 7.6) and treated with three different concentrations of plant extracts (10, 100 and 1000 ppm) in the final reaction volume of 15 µL. Fenton reaction was induced by addition of H_2O_2 (4 µL, 30%) and $FeSO_4$ (3 µL, 2mM). Four controls (untreated DNA, DNA treated with $FeSO_4$, DNA treated with H_2O_2, DNA treated with both $FeSO_4$ and H_2O_2) were run simultaneously. Samples were incubated at 37 °C in the dark for one hour. The reaction was stopped by addition of 2 µL 6X bromophenol blue. The samples were run on 1% agarose gel and visualized by a UV-transilluminator.

2.7 Bioactivity guided fractionation

The most potent extract, SN-L&F, was selected for conducting bioactivity-guided fractionation by based on its total phenolic content, DPPH radical scavenging and DNA protection activities.

2.7.1 Sample preparation

One gram of plant extract was dissolved in 100 ml of 80% methanol to get the final concentration of 10 mg/ml. The solution was washed three times with n-hexane (100 mL). Instrument grade water was added to get a final concentration of 5 mg/mL. The sample was filtered using GF/F followed by a 0.2 µm nylon syringe filter.

2.7.2 Semi-preparative HPLC fractionation

A semi-preparative gradient-elution HPLC method was developed for separating fractions. Fractionation was performed on an Alltima C18 column (10 mm × 250 mm; 5 µm) connected to an Alltima Prep-Guard C18 (7 mm × 33 mm; 10 µm) guard column. The chromatographic

system was composed of a Varian 9050 UV-vis detector, Varian 9012 pump system and a Gilson FC 203B fraction collector. Solvent A was a mixture of water/acetic acid (100:1, v/v), while solvent B was methanol/acetonitrile/acetic acid (90:10:1, v/v/v). Flow rate was 2 mL/min and the injection volume was 2 mL. The gradient started at 60 % solvent A and 40 % solvent B. Solvent B was increased to 60 % over 5 min and again to 65 % over 10 min. The gradient remained isocratic for 5 min and finally solvent B was increased to 70 % over 5 min. The system was equilibrated between runs for 10 min using starting mobile phase composition.. Fractions were collected at the following retention times: Fraction A= 0 min to 12.9 min; Fraction B= 13.0 min to 14.0 min; and Fraction C= 14.1 to 20.0 min.

Organic solvents were evaporated under vacuum at 45°C. Fractions were freeze-dried and stored at −20 °C. The purity of fractions was determined by analytical scale HPLC-DAD as described below.

2.7.3 Antioxidant activity of fractions

Antioxidant activities of fractions A, B and C were investigated by DPPH and Folin-Ciocalteu assay as described above.

2.8 Analytical scale HPLC

2.8.1 Sample preparation

Ten milligrams of plant extract powder were dissolved in 10 mL 80% methanol and then defatted by n-hexane (3 × 10 mL). Samples were filtered using GF/F filters and then through 0.2 µm nylon syringe filters.

224

225 *2.8.2 HPLC-diode array detection (HPLC-DAD)*

226 Analysis was performed on a Varian 9021 solvent delivery system equipped with Varian 9065
227 Polychrom UV-diode array detector (190-367 nm). The HPLC system was controlled using Star
228 Polychrom version 5.2 (Varian, Australian). The flow rate of 1 mL/min and injection volume of
229 20 µL were used. Sample analysis was performed by gradient elution on a 150 mm x 4.6 mm
230 I.D., 5 µM, Luna C-18(2) column (Phenomenex, Australia) with a SecurityGuard (Phenomenex,
231 Australia) guard cartridges. The mobile phases were freshly prepared, degassed under vacuum
232 using Phenomenex nylon 45 µm membranes and sonicated in a Sanophon ultrasonic bath
233 (Ultrasonic Industries Pty. Ltd, Australia) for 15 minutes prior to HPLC analysis. Solvent A was
234 a mixture of water/acetic acid (100+1, v/v), and solvent B was a mixture of
235 methanol/acetonitrile/acetic acid (90+10+1, v/v/v). A six-step gradient elution for a total run time
236 of 60 min was used as previously described (Obied et al., 2005).

237

238 *2.8.3 Liquid chromatography-mass spectrometry (LC-MS)*

239 LC-MS of the methanolic extracts of all plant species was performed on a Micromass Quattro
240 (Waters, Manchester, UK). LC separation was attained by a Waters liquid chromatograph
241 (Waters, Milford, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV
242 detector operated at 280 nm. Columns and gradients were same as described above for analytical
243 scale HPLC. An injection volume of 10 µL and a constant flow of 1 mL/min were used.. The
244 entire flow from the LC was directed to the mass spectrometer. Data were acquired by the
245 Masslynx data system for both the MS and UV data. The mass spectral data was acquired for four

scans i.e. Scan 1: positive ion mode, cone voltage 35 V; Scan 2: positive ion mode, voltage 70 V; Scan 3: negative ion mode, cone voltage 30 V; Scan 4: Negative ion mode, cone voltage 70 V.

2.9 Statistical analyses

Experiments were performed in triplicate. Data are presented as mean ± standard deviation qne were analysed by one-way ANOVA (SPSS version 11.1).

3. Results

3.1 Determination of total phenol content in crude extracts

Prior to measuring total phenols in the crude extract, it was necessary to determine if hexane washing removed any significant lipid soluble phenolic antioxidants. The UV-Vis spectrum of the crude extract before and after washing showed no change in absorbance in the two major bands associated with phenolic compounds, 280 nm, simple phenols; and 320 nm hydroxycinnamic acid derivatives. Spectra of the hexane washings showed only the expected features due to carotenoids and chlorophylls. For three extracts – *S. nubicola* stem (SN-S), *H. nepalensis* leaves and stem (HN-L&S), and *A. oblongifolium* leaves and stem (AO-L&S) – removal of pigments required three washes; while the leaves and flower extract of *S. nubicola* (SN-L&F) required four to five washes to remove the pigments.

Table 1 reports the total phenolic content of each of the crude extracts. The order of total phenolic content was SN-L&F > AO-L&S > SN-S > HN-L&S. There was about a 65% relative difference between the extracts with the lowest (HN-L&S) and the highest (SN-L&F) phenol

content. There was no clear relationship between the mass of crude extract per gram of dry weight of material and total phenolic content although generally the higher the mass of crude extract the higher the total phenols.

3.2 Phenolic profile of crude extracts

Fig. 1 shows the 280 nm chromatograms of the four crude extracts. The chromatograms of SN-L&F and SN-S (Figs. 1 a and b respectively) are dominated by rosmarinic acid. The chromatogram of HN-L&S (Fig. 1 c) shows more peaks, with chlorogenic acid and rutin predominant. For AO-L&S, the chromatogram consists of numerous small peaks, none of which could be identified conclusively (Fig. 1 d).

3.3 Antioxidant assays of crude extracts

3.3.1 DPPH radical scavenging activity

The DPPH radical scavenging activities of the four crude extracts were determined and the results are shown in Table 1 as mM TE/g extract. The order of the antioxidant activity of the extracts was SN-L&F > AO-L&S > SN-S > HN-L&S, which is the same order as found for the total phenol content. The relative difference in values was 89%,.

3.3.2 ABTS radical scavenging activity

The $ABTS^{\bullet+}$ scavenging activities of the four crude extracts were determined and the results are reported in Table 1 as mM TE/g extract. For this assay AO-L&S had highest activity followed by SN-L&F, then SN-S, and HN-L&S. As with the DPPH antioxidant activity, SN-S and HN-L&S

were the least effective antioxidants – they also had the least phenolic content (Table 1). The relative difference in values was 66% similar to the result for total phenol content, above.

3.3.3 TBARS assay

The TBARS antioxidant activity of the four crude extracts was determined and the results are reported in Table 1 as mM TE/g extract. The values ranged from 392.9 ± 3.42 (HN-L&S) to 462.1 ± 3.62 (AO-L&S). The relative difference in TBARS antioxidant activity among the extracts was only 15%, however, the activities were statistically significantly different from each other ($p < 0.05$).

3.3.4 DNA protection assay

DNA protection activity can be determined by observing the breakdown of supercoiled plasmid DNA by a mixture of hydrogen peroxide and Fe(II) (Fenton chemistry, Obied et al. 2009). As shown in Fig. 2, hydrogen peroxide alone has little effect on plasmid DNA (*cf*. lanes 2 and 4), whereas Fe(II) induces a single stand break leading to the formation of open circular plasmid DNA (lane 3). The combination of Fe(II) and hydrogen peroxide cleaves the DNA to a fairly uniform size of short strands as evidenced by the spot near the bottom of the plate (lane 5). All crude extracts at all concentrations tested were able to protect DNA from this type of cleavage. On the other hand, some extracts were unable to prevent single strand breaks: SN-L&F at 1000 ppm (lane 8); SN-S at 10 ppm (lane 9); HN-L&S at 10 and 1000 ppm (lanes 12 and 14); and all concentrations of AO-L&S (lanes 15-17, although some protection from single strand cleavage may be evident for the 100 ppm extract, lane 16). Effective protection for DNA cleavage was

provided by SN-L&F, 10 and 100 ppm; SN-S, 100 and 1000 ppm; HN-L&S 100 ppm. There were no consistent dose-response trends for any of the extracts.

3.4 Bioactivity guided fractionation

Consistently, the crude extract SN-L&F had the highest values for antioxidant activity in multiple assays and also had the highest total phenolic content. Therefore, this extract was chosen for bioactivity guided fractionation. Semi-preparative HPLC was used to produce three fractions denoted as Fraction A, Fraction B and Fraction C (Fig 3). Each 100 mg of extract yielded 72 mg of fraction A, 4 mg of fraction B and 14 mg of fraction C.

3.5 Antioxidant activity of fractions

3.5.1 Total phenolic content:

Total phenolic content of Fractions A, B, and C are listed in Table 2. Fraction B had the highest concentration of phenolic compounds, 2524.4 ± 3.90 mM of Trolox/g of fraction.

3.5.2 DPPH radical scavenging activity:

Fraction B was the most effective fraction in the DPPH assay with a radical scavenging activity of 367.9 ± 0.1 mM TE/g of fraction.

3.5.3 DNA protection assay:

DNA protection activity was demonstrated in all the fractions tested (Fig. 4), although there was variability in the level of activity at different concentrations of the fractions. As with the crude

extract, all fractions at all concentrations protected DNA from complete fragmentation, but not all fractions/concentrations could prevent single strand breaks.

In the case of fraction A, variable levels of DNA protection were observed at different concentrations (Fig 4. (a)), with 20 ppm, 40 ppm and 50 ppm being effective. For fraction B, DNA protection was highest at the two lowest concentrations, 1 ppm and 3 ppm, with no protection evident at 5, 7 and 9 ppm (Fig 4 (b)). However for fraction C, there was no apparent change in the level of activity at all concentrations (Fig 4 (c)).

3.6 Identification of phenolic compounds

Rosmarinic acid was identified by a combination of UV spectroscopy and mass spectrometry. The UV spectrum extracted from the HPLC-DAD matched that of an authentic standard and in the mass spectrum, the observation of the molecular ions at m/z 359 in the negative ion mode and m/z 361 in the positive ion mode are consistent with a molecular weight of 360 amu. The fragmentation pattern was consistent with that reported in the literature (Matsuda, 2010) with peaks at m/z 197, 179, 161, 135, 133 and 123 in the negative ion spectrum. Rutin and chlorogenic acid were confirmed in extracts through retention time and mass spectra of authentic standards.

4. Discussion

4.1 Total phenol content and antioxidant activity of crude extracts

Previous work by Inayatullah et al. (Inayatullah et al., 2007) demonstrated the bioactivity of a number of crude extracts from Pakistani traditional medicines, *viz*, leaf and flower extract of *S. nubicola* (SN-L&F); stem extract of *S. nubicola* (SN-S); leaf and stem extract of *H. nepalensis*

(HN-L&S); and leaf and stem extract of *A. oblongifolium* (AO-L&S). The aim of this work was to bioprospect these extracts to determine the one with the most potent antioxidant activity. As recommended by Frankel and Meyer (Frankel et al., 2000), multi-dimensional antioxidant assays were used to select the extract with highest antioxidant activity for further investigation. Bioactivity guided fractionation was then used to locate the compound(s) with highest antioxidant activity within the crude extract.

This work revealed a close association between total phenol content in the four extracts and antioxidant activity as measured by the DPPH radical and $ABTS^{\bullet+}$ scavenging assays. Thus SN-L&F had the highest total phenolic content and was the best antioxidant in these assays. This may be attributed to the fact that the determinations were conducted in hydrophilic matrices. In the lipophilic system, i.e. the TBARS assay, AO-L&S was the best performed antioxidant, but it still had the second highest total phenols. However, it may be argued that the TBARS assay (at least in this instance) had limited ability to differentiate the antioxidant potential because there was only a 15% difference between the lowest (SN-S) and highest (AO-L&S) values. This compares to differences in the order of 65% for the hydrophilic-based assays.

Previous work on other species of *Salvia* has shown that they are good sources of phenolic compounds (Akkol et al., 2008; Kosar, Goger & Baser, 2008) and have significant antioxidant activity using the DPPH radical scavenging assay (Bozan, Ozturk, Kosar, Tunalier & Baser, 2002; Gulcin, Uguz, Oktay, Beydemir & Kufrevioglu, 2004; Orhan et al., 2007). As far as the authors are aware, this is the first study to look at the antioxidant activity of *S. nubicola* in crude extracts of various plant parts. However, the antioxidant activity of SN-L&F appears comparable

387 to that reported in a study of 14 *Salvia* species from Turkey (Orhan et al., 2007). In the latter
388 study (Orhan et al., 2007), high DPPH scavenging activity was found in ethyl acetate and
389 methanol fractions.
390
391 There was a significant difference ($p < 0.05$) between the total phenolic content and antioxidant
392 activities of the different extracts of *S. nubicola*. The stem extract, SN-S was low in total phenols
393 (compared to SN-L&F) and low in antioxidant activities in all assays, except TBARS, where it
394 was second-most active. This is the first time (as far as the authors are aware) that the different
395 parts of a *Salvia* species have been examined for antioxidant activity and demonstrates that
396 significant partitioning of phenolic compounds occurs between the stem, leaves and flowers.
397
398 SN-L&F was the only extract to show DNA protection activity at the lowest concentration
399 measured (10 ppm). DNA protection activity at low concentration is consistent with the results of
400 other assays for SN-L&F. Furthermore, the results from the DNA protection assay confirm other
401 findings (Inayatullah et al., 2007) that *S. nubicola* is a known anticancer and antitumor plant
402 species It would appear likely that the high total phenolic content is responsible for protecting
403 DNA in this assay.
404
405 This is the first report (as far as the authors are aware) of the total phenolic content and
406 antioxidant activity of *H. nepalensis*. The present study revealed that HN-L&S had the lowest
407 antioxidant potential of all extracts tested. However, *H. nepalensis* is a known anticancer and
408 cytotoxic plant species as reported by Inayatullah et al. (Inayatullah et al., 2007). Perhaps the
409 bioactivity of HN-L&S is located more in its lipophilic components as HN-L&S had good

activity in the TBARS assay, only 15% less than the highest activity measured. Synergistic interactions among compounds in the crude extract may also be important for the bioactivity of this extract. There was no clear dose-response relationship for HN-L&S in the DNA protection assay. As *H. nepalensis* has been reported as an anti-diabetic (Gilani et al., 2001) further studies on this species may be better focused on protein binding assays, e.g. *β*-glucosidase.

Acer genera have been reported to be efficacious against cancer, polio, and dysentery (Moerman, 1998), and previously antioxidant activities and various phenolic compounds for a number of *Acer* species have been reported (Jiang et al., 2006). However, there are no reports on the antioxidant activity of *A. oblongifolium*. AO-L&S was consistently the second most potent antioxidant and had the second highest concentration of phenolic compounds of the extracts tested in this study. There appeared to be some DNA protection at 100 ppm in the DNA protection assay. Further work on bioactivities of *A. oblongifolium* is justified based on the levels of phenolic compounds found in AO-L&S in this study, and the lack of studies on this species in the literature.

4.2 Fractionation of SN-L&F crude extract and identification of phenolic compounds

Based on the total phenolic content of SN-L&S and its potency in the antioxidant activity assays, this extract was chosen for bioactivity guided fractionation to identify compounds with significant antioxidant activity. The division of the fractions was based on the 280 nm HPLC chromatogram (Fig 3), which showed one main peak. Thus fraction A was chosen to investigate early eluting, more polar compounds, most of which did not absorb at 280 nm. Fraction B was chosen to encompass the compound(s) eluting as the major peak in the chromatogram, while

fraction C was chosen to investigate the later eluting, less polar compounds (again, most of which did not absorb at 280 nm). The masses of material recovered from each fraction (Table 2), revealed that most of mass in the crude extract (96 %) is located in Fractions A (72%) and C (14%), or not recovered (10%). Fractions A and C did not give rise to peaks that absorbed strongly in the 280 nm chromatogram therefore those fractions are not composed of simple phenolic compounds to any great extent. Correspondingly, most of the crude extract is not composed of simple phenolic compounds. However, non-phenolic antioxidants may have been present in the crude extract and therefore it was important to test each fraction, and not concentrate solely on the largest peak in the chromatogram.

In determining the bioactivity of the various fractions, the DPPH radical scavenging assay and the DNA protection assay were chosen. The results of antioxidant assays with the crude extracts showed that the DPPH radical and $ABTS^{\bullet+}$ scavenging assays yielded complementary results, whereas the TBARS assay did not provide as much discrimination among the extracts as the non-lipid assays. Moreover, the very low content of total phenols in fractions A and C, compared to fraction B (Table 2), meant that it was unlikely that potent antioxidants would be uncovered through multidimensional testing. Hence a limited number of assays were used in the bioguided fractionation step.

The results of the DPPH assay showed that the majority of the antioxidant activity in SN-L&F arose from Fraction B (Table 2), which also had the highest content of total phenols. Thus there were no apparent potent, non-phenolic antioxidants in SN-L&F. On the other hand, all fractions

had some DNA protection activity (Fig. 4), but the most potent activity came from low concentrations (1 and 3 ppm) of Fraction B.

Analytical scale HPLC and LC-MS revealed the presence of rosmarinic acid (fraction B) as the major antioxidant in SN-L&F (Fig. 1). Rosmarinic acid also gave rise to the major peak in the 280 nm chromatogram of SN-S. Previous work (Akkol et al., 2008; Kosar et al., 2008) identified rosmarinic acid in many *Salvia* species as the major antioxidant. For *H. nepalensis*, chlorogenic acid and rutin were identified in HN-L&S (Fig. 1). These compounds are well-known potent antioxidants, but levels in HN-L&F were not sufficient for this extract to be considered for bioactivity guided fractionation studies. No known phenolic compounds could be identified in AO-L&S. Since this extract had good antioxidant activity, future work could investigate the compounds responsible.

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Figure Captions

Fig 1: 280 nm HPLC chromatograms for (a) SN-L&F, (b) SN-S, (c) HN-L&S, and (d) AC-L&S.

Fig 2: DNA protection assay. Lane 1 is λ Hind III marker. Lane 2-5 presents controls (untreated DNA, DNA treated with 2mM $FeSO_4$, DNA treated with 30 % H_2O_2, DNA treated with 2mM $FeSO_4$ and 30 % H_2O_2). Lane 6-8, DNA treated with SN-L&F at 10, 100 and 1000 ppm. Lane 9-11, DNA treated with SN-S at 10, 100 and 1000 ppm. Lane 12-14, DNA treated with HN-L&S at 10, 100 and 1000 ppm. Lane 15-17, DNA treated with AO-L&S at 10, 100 and 1000 ppm.

Fig 3: 280 nm HPLC chromatogram of SN-L&F showing the three fractions A, B and C in semi-preparative HPLC.

Fig 4: DNA protection assay with different concentrations of (a) fraction A, (b) fraction B and (c) fraction C of SN-L&F. Lane 1 is λ Hind III marker. Lane 2-5 presents controls (untreated DNA, DNA treated with 2mM $FeSO_4$, DNA treated with 30 % H_2O_2, DNA treated with 2mM $FeSO_4$ and 30 % H_2O_2). In (a) and (c), lanes 6-10 show DNA treated with 0, 20, 30, 40 and 50 ppm of fraction A and C. In (b) lanes 6-10 show DNA treated with 1, 3, 5, 7 and 9 ppm of fraction B.

Table 1. Extractable material, total phenol content and antioxidant activity of crude extracts (difference in superscript letters indicate significance level at $p < .05$)

Plant Extracts	Extractable material (mg of crude extract/g dry weight)	Total phenols (mg GAE/g extract)	Total phenols (mM TE/g extract)	DPPH radical scavenging activity (mM TE/g extract)	$ABTS^{\bullet+}$ scavenging activity (mM TE/g extract)	TBARS (mM TE/g extract)
SN-L&F	80	138.8 ± 0.2^{a}	342.1 ± 19.8^{a}	248.4 ± 0.5^{a}	149.2 ± 3.5^{b}	400.3 ± 6.9^{c}
SN-S	30	82.5 ± 0.3^{c}	$187.5 \pm 1 2.80^{c}$	66.9 ± 0.8^{c}	70.4 ± 2.6^{c}	420.1 ± 8.9^{b}
HN-L&S	20	47.7 ± 0.3^{d}	107.9 ± 3.83^{d}	26.7 ± 0.2^{d}	54.7 ± 1.6^{d}	392.9 ± 3.4^{d}
AO-L&S	200	104.4 ± 0.5^{b}	233.0 ± 13.05^{b}	140.4 ± 1.2^{b}	160.2 ± 6.4^{a}	462.1 ± 3.6^{a}

Table 2: Mass, total phenolic content and DPPH radical scavenging activity of fractions A, B and C of SN-L&F extract (difference in letters present significance at $p < .05$).

	Mass of fraction per 100 mg crude extract (mg)	Total phenolic content (mM Trolox/gram of fraction)	DPPH scavenging activity (mM TE/gram of fraction)
Fraction A	72	1279.2 ± 0.7^{c}	73.3 ± 0.1^{c}
Fraction B	4	2524.4 ± 3.9^{a}	367.9 ± 0.1^{a}
Fraction C	14	1395.6 ± 3.6^{b}	173.7 ± 0.1^{b}

Figure 1

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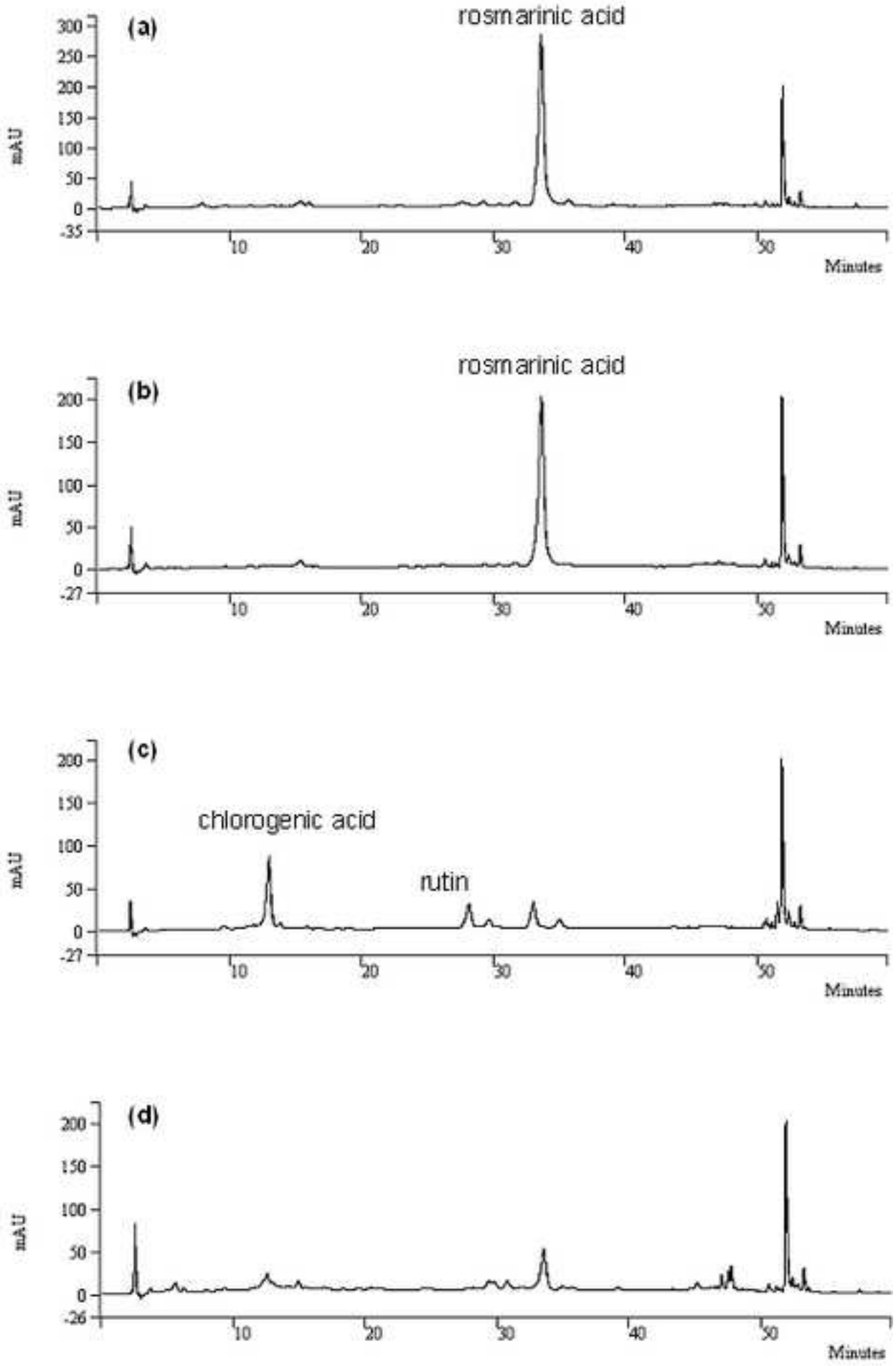

Figure 2

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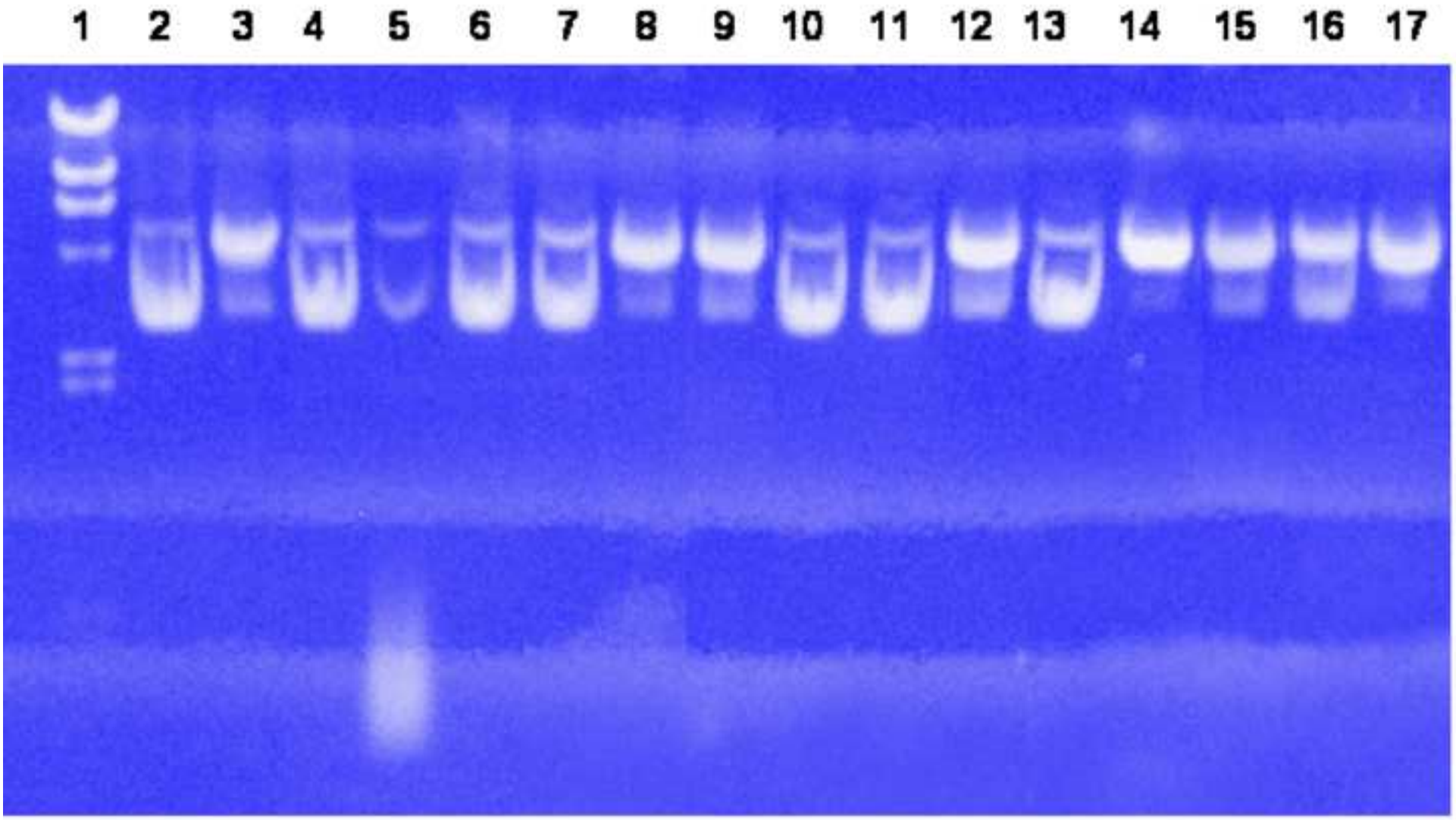

Figure 3

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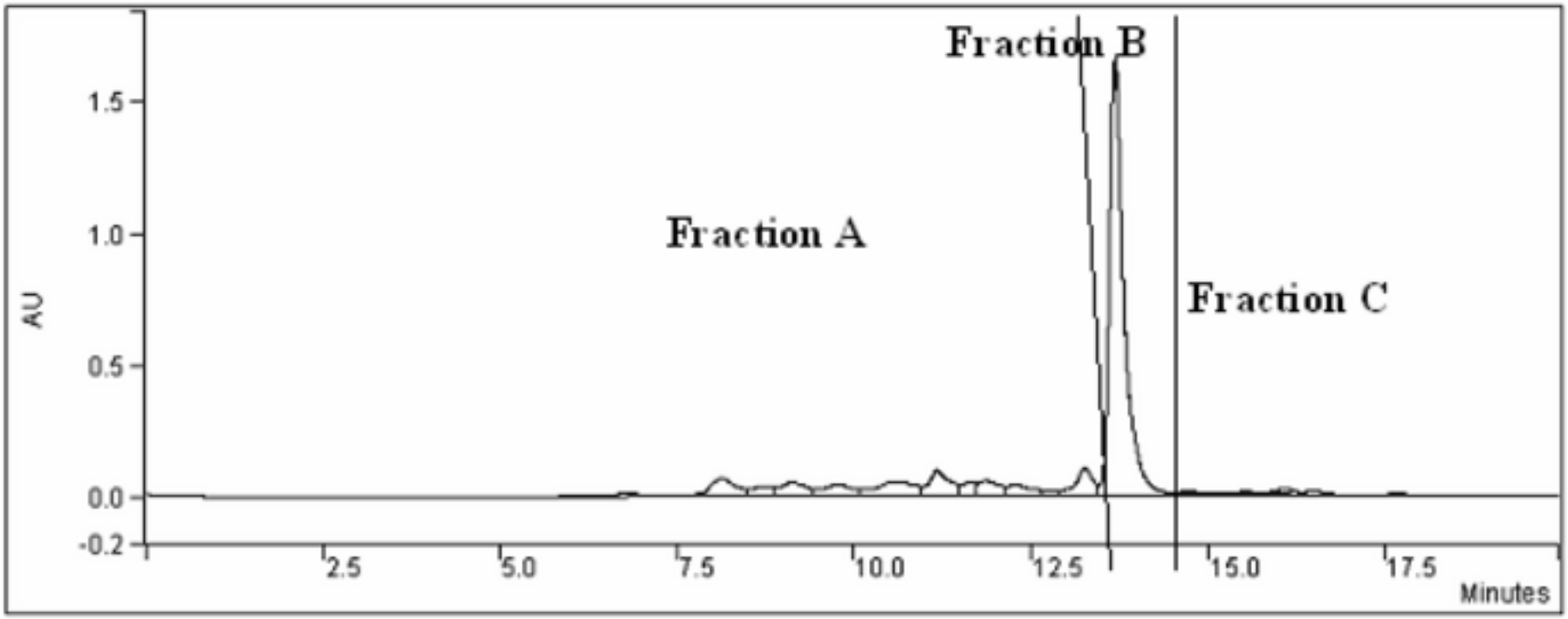

Figure 4

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