Khan, KM, Nahar, L, Mannan, A, Arfan, M, Khan, GA, Hobbs, G and Sarker, SD

Evaluation of Resazurin Microtiter Plate Assay and HPLCPhotodiode Array Analysis of the Roots of Asparagus adscendens

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**Evaluation of Resazurin Microtiter Plate Assay and HPLC-Photodiode Array Analysis of the Roots of Asparagus adscendens**

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| Complete List of Authors: | khan, kashif; COMSATS Institute of Information Technology - Abbottabad, Pharmacy  
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Mannan, Abdul; COMSATS Institute of Information Technology - Abbottabad, Department of Pharmacy  
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| Keywords: | Asparagus adscendens, Asparagaceae, SPE, REMA, MIC, HPLC-PDA |

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Evaluation of Resazurin Microtiter Plate Assay and HPLC- Photodiode Array Analysis of the Roots of *Asparagus adscendens*

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**ABSTRACT**

*Asparagus adscendens* Roxb. (Asparagaceae), is native to the Himalayas. The present study, for the first time, was undertaken to explore the antimicrobial potential, to determine the minimum inhibitory concentration (MIC) values of the methanol extract of the roots of *Asparagus adscendens* and its solid phase extraction (SPE) fractions by using resazurin microtiter assay (REMA) against gram positive and negative bacterial registered strains and to carry out HPLC-Photodiode array analysis of the SPE fractions. The methanol extract and all SPE exhibited considerable level of antibacterial potential against gram-positive bacteria (MIC: 2.5-0.009 mg/mL) than against gram-negative bacteria (MIC: 1.25-2.5 mg/mL). The use of microtiter plates has the advantage of lower cost, fast and quantitative results. Like other *Asparagus* species, the presence of phenolic compounds in all SPE fractions was evident in the HPLC-PDA data.

**Keywords:** *Asparagus adscendens*; Asparagaceae; SPE; REMA; MIC; HPLC-PDA

1. Introduction

*Asparagus adscendens* Roxb. (Asparagaceae), commonly known as “safed musli” in Pakistan and various common names in India i.e. Shatawari, Shatavar, Shathamuli, Sahasrapal, Sainsarbuti, is native to Himalayas (Mannan et al., 2015). The genus *Asparagus* comprises about 300 species, and most of the European species are used as vegetables (Goyal et al., 2003; Singh and Geetanjali, 2016). Of the species that grow in the Himalayan region of Pakistan, *A. adscendens* and *A. racemose*, are the most commonly used species in traditional medicines. Aliphatic, nitrogenous and phenolic compounds, saponins, steroids and triterpenoids have been reported from *A. adscendens* of Indian origin (Mannan et al., 2015); β-sitosterol glucoside, spirostanol glycosides (asparanin C and asparanin D) and furostanol glycosides (asparoside C and asparoside D) were isolated by (Sharma et al., 1982); steroidal saponins and glycosides, and various lipophilic compounds were found in the tuberous roots and leaves, sarsasapogenin, diosgenin, β-sitosterol glucoside, spirostanol glycosides (asparanin A and B) and furostanol glycosides (asparaside A and B), were isolated from this plant (Jadhav and Bhutani, 2006; Sharma et al., 1980).
Colorimetric methods of drugs susceptibility testing produce results more quickly than standard culture methods (Martin et al., 2007) and are less costly than molecular methods (Katawera et al., 2014). For example the resazurin microtiter assay (REMA), which relies on an oxidation-reduction reaction to induce a blue to pink colour change in the presence of live bacteria (Fig.1), (Pfaller et al., 1994). HPLC-PDA detection has grown into one of the frequently used technique for analysing the herbs (Cai et al., 2006). Photodiode array (PDA) detector can collect multiple wavelength of chromatograms and corresponding spectra at the same time. The purity of the chromatographic peaks can also be determined and it can be used for spectra retrieval. Moreover, the qualitative information of chemical constituents can be obtained (Tan et al., 2014). Therefore, the present study was undertaken to explore the antimicrobial potential, to determine the minimum inhibitory concentration (MIC) values of the MeOH extract of the roots of Asparagus adscendens and its SPE fractions by using resazurin microtiter assay (REMA) against Gram positive and negative bacterial registered strains and to carry out HPLC-PDA analysis of the SPE fractions.

2. Results and discussion

2.1 Resazurin assay

The MeOH extract of the roots of Asparagus adscendens and its SPE fractions (AAMF1, AAMF2, AAMF3 & AAMF4) exhibited varying antibacterial activity by using resazurin microtiter plate assay as shown in (Table S1). The MIC (mg/mL) values of the MeOH extract and its SPE fractions are shown in Table S1. The results from (Table S1) indicated that the MeOH extract (AAM) and four SPE fractions showed higher antibacterial activity against Gram-positive bacteria (MIC: 2.5-0.009 mg/mL) than against Gram-negative bacteria (MIC: 1.25-2.5 mg/mL). The SPE fraction AAMF1, which had the most polar components of the parent MeOH extract, showed most significant antibacterial activity against M. luteus (MIC: 0.078 mg/mL), and considerable antibacterial activity against S. aureus, B. subtilis, E. coli and K. oxytoca (MIC: 2.5, 2.5, 2.5 and 2.5 mg/mL respectively). The SPE fraction AAMF2 showed most prominent antibacterial activity against B. subtilis and M. luteus (MIC: 0.31 and 0.31 mg/mL respectively), and showed mild activity against S. aureus, E. coli and K. oxytoca (MIC: 1.25, 2.5 and 2.5 mg/mL respectively). The SPE fraction AAMF3 showed most significant antibacterial activity against S. aureus, B. subtilis and M. luteus (MIC: 0.625, 0.0195 and 0.078 mg/mL respectively) and showed mild antibacterial activity against E. coli and K. oxytoca (MIC: 2.5 and 1.25 mg/mL respectively) was quite similar to that of the
AAMF1, AAMF2 and the MeOH extract. The SPE fraction AAMF4 which contained the least polar components of the parent MeOH extract, exhibited notable antibacterial activity against *S. aureus*, *B. subtilis* and *M. luteus* (MIC: 0.156, 0.009 and 0.156 mg/mL) and showed no activity against *E. coli* and *K. oxytoca* (MIC: ≥10). This is the first report to explore the antimicrobial potential to determine the minimum inhibitory concentration (MIC) values of the MeOH extract of the roots of *Asparagus adscendens* and its SPE fractions by using resazurin microtiter assay (REMA) against Gram positive and negative bacterial strains. The use of microtiter plates has the advantage of lower cost, fast and quantitative results.

2.2. Performance Liquid Chromatography –Photodiode array detection

HPLC-PDA analysis on the SPE fractions of the MeOH extract of the roots of *A. adscendens* was performed to obtain insights into the possible chemical composition of the fractions, particularly, to have an indication whether they contain phenolic and flavonoids as possible contributors to the significant antibacterial activity of the extract and its fractions. The chromatographic conditions were optimized by method development. A linear gradient elution with water and MeOH containing 1% TFA as the mobile phase offered the best resolution. Typical chromatograms of fractions (Fig. S2) were recorded by using PDA detector at 220, 254, 360 nm to provide a real time chromatograms and on-line Ultraviolet (UV) spectra from 200-500 nm were recorded (Table S2) for identification of different groups and classes of compounds. The possible presence of compounds such as phenolic compounds, that might be Flavonoids or Isoflavoinoids in all SPE fractions could be suggested from the retention times and the UV-vis spectral data of the separated peaks. The consistency of the chromatograms of these fractions reflects the similar chemical constituents.

It was observed that the most compounds in the chromatograms (Fig. S2) possessed strong UV absorption at different retention time (*t*<sub>R</sub> in min.). Previous studies revealed that different phytochemicals like steroids, triterpenoids, glycosides, saponins, phenolic compounds, aliphatic compounds, alkaloids, tannins and nitrogenous constituents reported in these plants (Manta *et al.*., 1995; Thakur and Sharma, 2015). Spirostanol glycosides (asparanin A and asparanin B) and two furostanol glycosides (asparoside A and asparoside B) have been isolated from the methanol extract (Jadhav and Bhutani, 2006). From one of the previous published data (Liang tan et al), 280nm was chosen as the detection wavelength for
epicatechin and 360nm for various flavonoids such as myricetin, hyperoside, quercitrin and quercetin.

3. Experimental
See Supplementary materials.

4. Conclusion

Presence of phenolic compounds like epicatechin and various flavonoids such as myricetin, hyperoside, quercitrin and quercetin in *A. adscendens* was in agreement with that of other *Asparagus* species. It is reasonable to assume that the antibacterial activity of the MeOH extract and its SPE fractions of the roots of *A. adscendens* might be, at least partly, owing to the presence of phenolic compounds and could be potential source of antimicrobial compounds. This is the first report, on the preliminary HPLC-PDA analysis on *A. adscendens*.

Disclosure statement

The authors have declared that there is no conflict of interest.

References


Supporting Information

Evaluation of Resazurin Microtiter Plate Assay and HPLC- Photodiode Array Analysis of the Roots of *Asparagus adscendens*

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**Keywords:** *Asparagus adscendens*; Asparagaceae; SPE; REMA; MIC; HPLC-PDA
Experimental

Reagents and chemicals

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich (Dorset, UK). Solvents were purchased from Fisher Scientific (Loughborough, UK); sterile resazurin tablets (Fischer Chemicals); UV spectrophotometer (Shimadzu); HPLC Agilent 1260 Infinity; Incubator (Binder) at 35 and 37 °C; vortex mixer (Labnet International); Eppendorf Centrifuge 5810 R (Fischer Scientific, UK); Shaker Incubator (Sartorius CERTOMAT); Four NCTC and One ACTC registered bacterial strains were obtained from the Microbiology labs of School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University.

Plant materials. Plant sample was collected from Muzaffarabad district of Pakistan-controlled Azad Kashmir and identified as Asparagus adscendens Roxb. by Dr Muhammad Zafar, Herbarium Botanist, Department of Plant Sciences, Quaid-I-Azam University, Islamabad, Pakistan. A herbarium specimen for this collection (voucher number: Acc no. PAC1001) has been deposited and retained in the above herbarium.

Extraction and preparation of plant samples. Shade-dried and finely ground roots (2.5 kg) of Asparagus adscendens were macerated in MeOH (5 L) for 10 days at room temperature, filtered, and the solvent was evaporated under vacuum using a rotatory evaporator (<45oC) to obtain concentrated gummy crude extract.

Solid-phase extraction (SPE) and sample purification. A portion of the dried MeOH extract (2 g) was suspended in 20 mL of HPLC grade water and loaded on to a Strata C-18 cartridge (20 g), previously washed with MeOH (50 mL) followed by equilibration with water (100 mL). The cartridge was eluted with MeOH-water mixture of decreasing polarity to obtain...
four fractions: 20, 50, 80 and 100% MeOH in water (250 mL each), coded respectively as AAMF1, AAMF2, AAMF3 and AAMF4. All four fractions were evaporated to dryness using a combination of rotary evaporator and freeze-dryer, re-dissolved in MeOH (10 mg/mL), centrifuged at 12,000 rpm for 3 min, filtered through 0.20 µm sterile syringe filter for injection (10 µL) into the HPLC-PDA system.

**High Performance Liquid Chromatography –Photodiode array detection (HPLC-PDA)**

An analytical Agilent 1260 Infinity was used. Reversed-phase chromatography was performed on a Phenomenex Gemini-NX 5 U C18 column (250 x 4.6 mm). The column temperature was set at 25°C. A variable wavelength UV-Vis detector was set at 220 nm, 254nm and 360nm. An elution gradient was used with solvent A (1% trifluoroacetic acid in water) and solvent B (1% trifluoroacetic acid in MeOH). The initial mobile phase composition was 70% of A and 30% B at 0 min, then linear gradient to 100% of B over 30 min and held at that composition for 5 min before to returning to start conditions and column equilibration at flow rate of 0.800 mL/min. The chromatograms were monitored as 220 nm, 254 nm and 360 nm.

**Minimum inhibitory concentration (MIC) determination**

The *in vitro* susceptibility testing was performed using a 96-well microtiter plate with resazurin. A stock solution of antibiotics was prepared at a concentration of 128 µg/ml by dissolving in sterile distilled water. Plant extracts were prepared with 10% DMSO to obtain a stock concentration of 10 mg/ml (Sarker *et al.*, 2007). Sterile Distilled water was used to dissolve resazurin dye to obtain 0.02 % and the solution was then sterilized by filtration. The MIC assay was carried out in according to CLSI guideline for microdilution test (Clinical Laboratory Standards Institute, 2012). Briefly, the stock antibiotics and plant extracts were serially two fold diluted with cation-adjusted Mueller hinton broth (CAMHB). The additional
60 µL of the CAMHB and 20 µL of 0.02% resazurin were added to all wells. An Overnight culture of test bacteria was harvested by centrifugation at 4,000 rpm for 10 min, washed twice with NaCl by centrifugation at 4,000 rpm for 5 min each. The pellet collected was then adjusted approximately 0.5 standard McFarland equivalent (1 x 10⁸ CFU/mL), diluted to give 5 x 10⁶ CFU/mL, and then 20 µL will be transferred to the well so that the final concentration of inoculum is approximately 5 x 10⁵ CFU/mL. The total volume in each well was 200 µL and the final concentration of antibiotics and the extracts were 0.06-64 µg/mL and 0.005-5 mg/mL, respectively. Wells without antibacterial agents and bacterial strain were used as controls. The 96-well microplate was then incubated at 37 °C for 24 h. The lowest concentration showing no colorimetric change from blue (resazurin) to pink (resorufin) was noted as the MIC. Each test was carried out in triplicate. The average values were calculated for the MIC of test material.

Statistical analysis. The data were expressed as mean values ± standard error of the mean (SEM) of three parallel replicates. The graph was plotted using non-linear regression with the use of Microsoft Excel version 2013. The means were separated at confidence level p≤0.05 by using analysis of variance (ANOVA) with Tukey’s range test.

Results
Figure S1: HPLC-PDA Chromatogram of AAMF1 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.
Figure S2. HPLC-PDA Chromatogram of AAMF3 of *Asparagus adscendens* extract monitored at 254nm.
Figure S3: Corresponding UV-vis absorbance (AAMF1) at multiple wavelengths of the peaks separated by HPLC
Figure S4: HPLC-PDA Chromatogram of AAMF2 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.
**Figure S5**: Corresponding UV-vis absorbance (AAMF2) at multiple wavelengths of the peaks separated by HPLC-PDA
Figure S6: HPLC-PDA Chromatogram of AAMF3 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.
Figure S7: Corresponding UV-vis absorbance (AAMF3) at multiple wavelengths of the peaks separated by HPLC-PDA
Figure S8: HPLC-PDA Chromatogram of AAMF4 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.
Figure S9: Corresponding UV-vis absorbance (AAMF4) at multiple wavelengths of the peaks separated by HPLC-PDA.
Figure S10: Typical Plate after 24 h in resazurin assay [final concentration of antibiotics and the extracts were 0.06-64 µg/ml and 0.005-5 mg/ml, respectively; lowest concentration showing no colorimetric change from blue (resazurin) to pink (resorufin) was noted as the MIC; the test organism was *Bacillus subtilis* 1604; CTX (Cefotaxime), AMP (Ampicillin), AMX (Amoxicillin), AAM (*Asparagus adscendens* methanol extract)].
Table S1. The MIC (mg/mL) values of the MeOH extract of the roots of *A. adscendens* and its SPE fractions by using the resazurin assay

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<tr>
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<td>AAMF3 (mg/mL)</td>
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<td>AAMF4 (mg/mL)</td>
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</tr>
<tr>
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</tr>
<tr>
<td>AMP&lt;sup&gt;c&lt;/sup&gt; (µg/mL)</td>
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<tr>
<td>AMX&lt;sup&gt;d&lt;/sup&gt; (µg/mL)</td>
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SA, *Staphylococcus aureus* NCTC 7508; BS, *Bacillus subtilis* NCTC 1604; ML, *M. luteus* NCTC 7508; EC, *Escherichia coli* ATCC 25922; KO, *K. oxytoca* NCTC 8017; AAM<sup>a</sup> (*Asparagus adscendens* methanol extract), CTX<sup>b</sup> (*Cefotaxime*), AMP<sup>c</sup> (*Ampicillin*), AMX<sup>d</sup> (*Amoxicillin*)
**Table S2.** Retention times (t<sub>R</sub>) and corresponding UV-vis absorbance at multiple wavelengths of the peaks separated by HPLC of SPE fractions of the MeOH extract of the roots of *Asparagus adscendens*

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<sup>a</sup>Possible Phenolic Compounds
Dear Editor,

Thank you very much for your email and the referee’s comments. We have now carefully considered all comments of the referees and amended our manuscript as appeared to be essential and appropriate. Our response to referees; comments are shown below.

Also please note that all changes are highlighted in yellow on the manuscript. Figure 1, has submitted in supplementary data. I believe that the revised version of our manuscript is now acceptable for publication in the NPR as a short communication. I look forward to hearing from you in due course.

Yours faithfully, Kashif M Khan

Response to referee’s comments

Editorial Office:
Figure 1 regards experimental data and it should be submitted as supplementary material.
Response: Figure 1, has submitted in supplementary data

Reviewer: 1 Comments to the Author

I have reviewed the manuscript. There are some spelling mistakes. For example at the introduction section (β-sitosterol glucoside). These errors will be appropriate for the magazine when edited.

Response: Spelling mistakes have been thoroughly checked and highlighted in yellow after correction (β-sitosterol glucoside) on page 2 of introduction.