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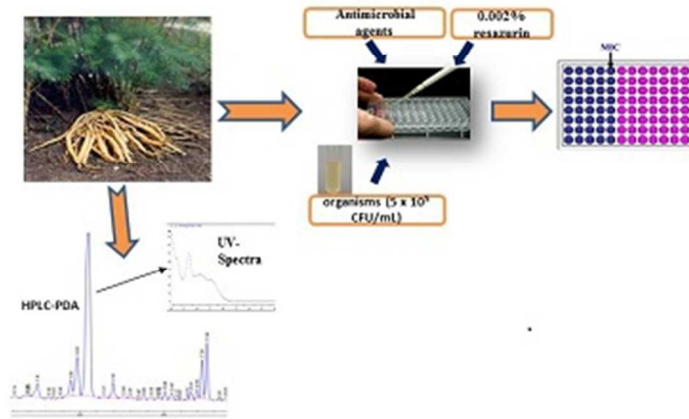
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Evaluation of Resazurin Microtiter Plate Assay and HPLC-Photodiode Array Analysis of the Roots of *Asparagus adscendens*

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Keywords:	Asparagus adscendens, Asparagaceae, SPE, REMA, MIC, HPLC-PDA

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Review Only

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3 **Evaluation of Resazurin Microtiter Plate Assay and HPLC- Photodiode**
4 **Array Analysis of the Roots of *Asparagus adscendens***
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44 of Pakistan] under Grant [PIN: IRSIP 28PS18].
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Evaluation of Resazurin Microtiter Plate Assay and HPLC- Photodiode Array Analysis of the Roots of *Asparagus adscendens*

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, Shatamuli, 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 (asparoside A and B), were isolated from this plant (Jadhav and Bhutani, 2006; Sharma *et al.*, 1980).

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3 Colorimetric methods of drugs susceptibility testing produce results more quickly
4 than standard culture methods (Martin *et al.*, 2007) and are less costly than molecular
5 methods (Katawera *et al.*, 2014). For example the resazurin microtiter assay (REMA), which
6 relies on an oxidation-reduction reaction to induce a blue to pink colour change in the
7 presence of live bacteria(Fig.1), (Pfaller *et al.*, 1994). HPLC-PDA detection has grown into
8 one of the frequently used technique for analysing the herbs (Cai *et al.*, 2006). Photodiode
9 array (PDA) detector can collect multiple wavelength of chromatograms and corresponding
10 spectra at the same time. The purity of the chromatographic peaks can also be determined and
11 it can be used for spectra retrieval. Moreover, the qualitative information of chemical
12 constituents can be obtained (Tan *et al.*, 2014). Therefore, the present study was undertaken
13 to explore the antimicrobial potential, to determine the minimum inhibitory concentration
14 (MIC) values of the MeOH extract of the roots of *Asparagus adscendens* and its SPE
15 fractions by using resazurin microtiter assay (REMA) against Gram positive and negative
16 bacterial registered strains and to carry out HPLC-PDA analysis of the SPE fractions.
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27 **2. Results and discussion**

28 **2.1 Resazurin assay**

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31 The MeOH extract of the roots of *Asparagus adscendens* and its SPE fractions (AAMF1,
32 AAMF2, AAMF3 & AAMF4) exhibited varying antibacterial activity by using resazurin
33 microtiter plate assay as shown in (Table S1). The MIC (mg/mL) values of the MeOH extract
34 and its SPE fractions are shown in Table S1. The results from (Table S1) indicated that the
35 MeOH extract (AAM) and four SPE fractions showed higher antibacterial activity against
36 Gram-positive bacteria (MIC: 2.5-0.009 mg/mL) than against Gram-negative bacteria (MIC:
37 1.25-2.5 mg/mL). The SPE fraction AAMF1, which had the most polar components of the
38 parent MeOH extract, showed most significant antibacterial activity against *M. luteus* (MIC:
39 0.078 mg/mL), and considerable antibacterial activity against *S. aureus*, *B. subtilis*, *E. coli*
40 and *K. oxytoca* (MIC: 2.5, 2.5, 2.5 and 2.5 mg/mL respectively).The SPE fraction AAMF2
41 showed most prominent antibacterial activity against *B. subtilis* and *M. luteus* (MIC: 0.31 and
42 0.31 mg/mL respectively), and showed mild activity against *S. aureus*, *E. coli* and *K. oxytoca*
43 (MIC: 1.25, 2.5 and 2.5 mg/mL respectively). The SPE fraction AAMF3 showed most
44 significant antibacterial activity against *S. aureus*, *B. subtilis* and *M. luteus* (MIC: 0.625,
45 0.0195 and 0.078 mg/mL respectively) and showed mild antibacterial activity against *E. coli*
46 and *K. oxytoca* (MIC: 2.5 and 1.25 mg/mL respectively) was quite similar to that of the
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3 AAMF1, AAMF2 and the MeOH extract. The SPE fraction AAMF4 which contained the
4 least polar components of the parent MeOH extract, exhibited notable antibacterial activity
5 against *S. aureus*, *B. subtilis* and *M. luteus* (MIC: 0.156, 0.009 and 0.156 mg/mL) and
6 showed no activity against *E. coli* and *K. oxytoca* (MIC: ≥ 10). This is the first report to
7 explore the antimicrobial potential to determine the minimum inhibitory concentration (MIC)
8 values of the MeOH extract of the roots of *Asparagus adscendens* and its SPE fractions by
9 using resazurin microtiter assay (REMA) against Gram positive and negative bacterial
10 strains. The use of microtiter plates has the advantage of lower cost, fast and quantitative
11 results.
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19 **2.2. Performance Liquid Chromatography –Photodiode array detection**

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21 HPLC-PDA analysis on the SPE fractions of the MeOH extract of the roots of *A. adscendens*
22 was performed to obtain insights into the possible chemical composition of the fractions,
23 particularly, to have an indication whether they contain phenolic and flavonoids as possible
24 contributors to the significant antibacterial activity of the extract and its fractions. The
25 chromatographic conditions were optimized by method development. A linear gradient
26 elution with water and MeOH containing 1% TFA as the mobile phase offered the best
27 resolution. Typical chromatograms of fractions (Fig. S2) were recorded by using PDA
28 detector at 220, 254, 360 nm to provide a real time chromatograms and on-line Ultraviolet
29 (UV) spectra from 200-500 nm were recorded (Table S2) for identification of different
30 groups and classes of compounds. The possible presence of compounds such as phenolic
31 compounds, that might be Flavonoids or Isoflavonoids in all SPE fractions could be
32 suggested from the retention times and the UV-vis spectral data of the separated peaks. The
33 consistency of the chromatograms of these fractions reflects the similar chemical
34 constituents.
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46 It was observed that the most compounds in the chromatograms (Fig. S2) possessed
47 strong UV absorption at different retention time (t_R in min.). Previous studies revealed that
48 different phytochemicals like steroids, triterpenoids, glycosides, saponins, phenolic
49 compounds, aliphatic compounds, alkaloids, tannins and nitrogenous constituents reported in
50 these plants (Manta *et al.*, 1995; Thakur and Sharma, 2015). Spirostanol glycosides
51 (asparanin A and asparanin B) and two furostanol glycosides (asparoside A and asparoside B)
52 have been isolated from the methanol extract (Jadhav and Bhutani, 2006). From one of the
53 previous published data (Liang tan et al), 280nm was chosen as the detection wavelength for
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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.

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9 and HPLC–PDA–ESI/MSn. *Arabian J. Chem.* DOI: org.10.1016/j.arbjc.2014.10.038
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Supporting Information

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

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 (<45°C) 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

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3 four fractions: 20, 50, 80 and 100% MeOH in water (250 mL each), coded respectively as
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5 AAMF1, AAMF2, AAMF3 and AAMF4. All four fractions were evaporated to dryness using
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7 a combination of rotary evaporator and freeze-dryer, re-dissolved in MeOH (10 mg/mL),
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9 centrifuged at 12,000 rpm for 3 min, filtered through 0.20 μ m sterile syringe filter for
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11 injection (10 μ L) into the HPLC-PDA system.
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13 14 ***High Performance Liquid Chromatography –Photodiode array detection (HPLC-PDA)***

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17 An analytical Agilent 1260 Infinity was used. Reversed-phase chromatography was
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19 performed on a Phenomenex Gemini-NX 5 U C₁₈ column (250 x 4.6 mm). The column
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21 temperature was set at 25°C. A variable wavelength UV-Vis detector was set at 220 nm,
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23 254nm and 360nm. An elution gradient was used with solvent A (1% trifluoroacetic acid in
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25 water) and solvent B (1% trifluoroacetic acid in MeOH). The initial mobile phase
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27 composition was 70% of A and 30% B at 0 min, then linear gradient to 100% of B over 30
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29 min and held at that composition for 5 min before to returning to start conditions and column
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31 equilibration at flow rate of 0.800 mL/min. The chromatograms were monitored as 220 nm,
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33 254 nm and 360 nm.
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37 38 ***Minimum inhibitory concentration (MIC) determination***

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41 The *in vitro* susceptibility testing was performed using a 96-well microtiter plate with
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43 resazurin. A stock solution of antibiotics was prepared at a concentration of 128 μ g/ml by
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45 dissolving in sterile distilled water. Plant extracts were prepared with 10% DMSO to obtain a
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47 stock concentration of 10 mg/ml (Sarker *et al.*, 2007). Sterile Distilled water was used to
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49 dissolve resazurin dye to obtain 0.02 % and the solution was then sterilized by filtration. The
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51 MIC assay was carried out in according to CLSI guideline for microdilution test (Clinical
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53 Laboratory Standards Institute, 2012). Briefly, the stock antibiotics and plant extracts were
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55 serially two fold diluted with cation-adjusted Mueller hinton broth (CAMHB). The additional
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3 60 μL of the CAMHB and 20 μL of 0.02% resazurin were added to all wells. An Overnight
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5 culture of test bacteria was harvested by centrifugation at 4,000 rpm for 10 min, washed
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7 twice with NaCl by centrifugation at 4,000 rpm for 5 min each. The pellet collected was then
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9 adjusted approximately 0.5 standard McFarland equivalent (1×10^8 CFU/mL), diluted to
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11 give 5×10^6 CFU/mL, and then 20 μL will be transferred to the well so that the final
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13 concentration of inoculum is approximately 5×10^5 CFU/mL. The total volume in each well
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15 was 200 μL and the final concentration of antibiotics and the extracts were 0.06-64 $\mu\text{g/mL}$
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17 and 0.005-5 mg/mL, respectively. Wells without antibacterial agents and bacterial strain were
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19 used as controls. The 96-well microplate was then incubated at 37 °C for 24 h. The lowest
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21 concentration showing no colorimetric change from blue (resazurin) to pink (resorufin) was
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23 noted as the MIC. Each test was carried out in triplicate. The average values were calculated
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25 for the MIC of test material.
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30 **Statistical analysis.** The data were expressed as mean values \pm standard error of the mean
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32 (SEM) of three parallel replicates. The graph was plotted using non-linear regression with the
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34 use of Microsoft Excel version 2013. The means were separated at confidence level $p \leq 0.05$ by
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36 using analysis of variance (ANOVA) with Tukey's range test.
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40 41 **Results**

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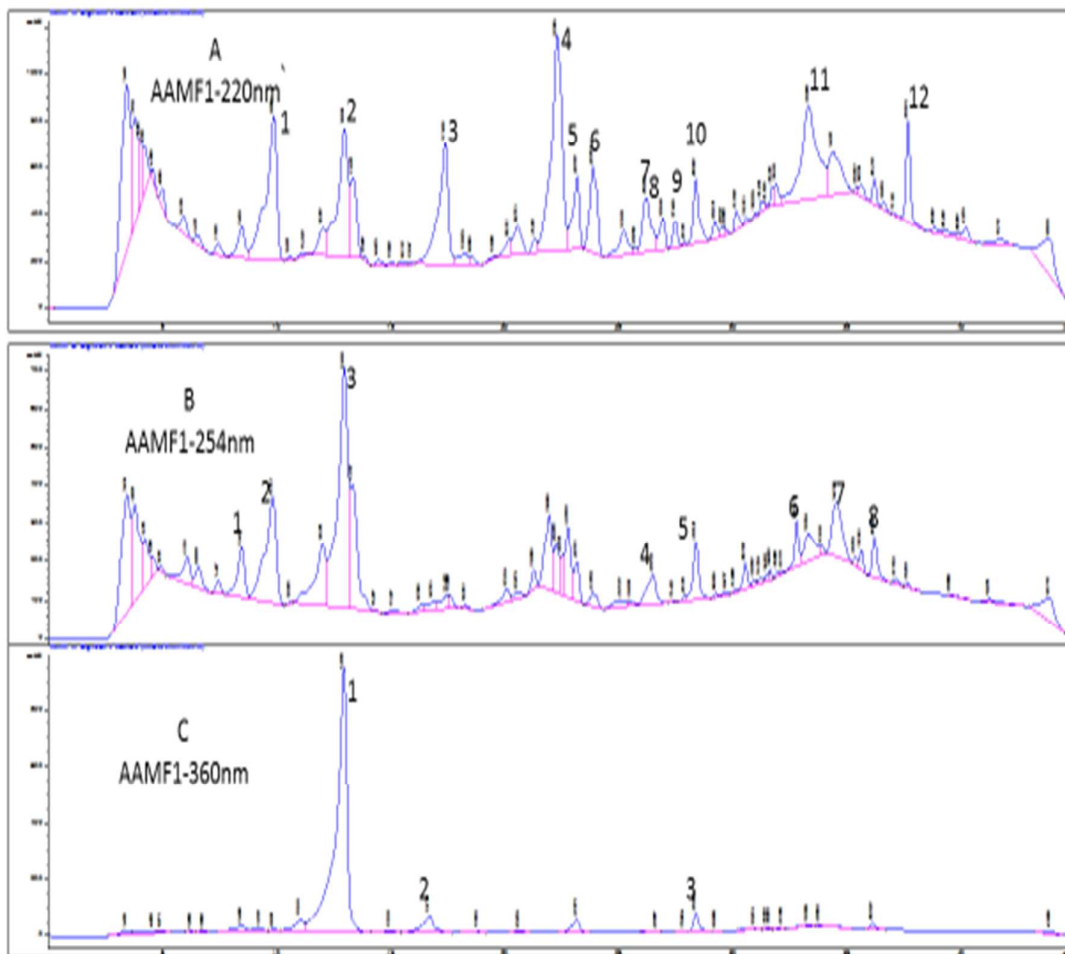


Figure S1: HPLC-PDA Chromatogram of AAMF1 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.

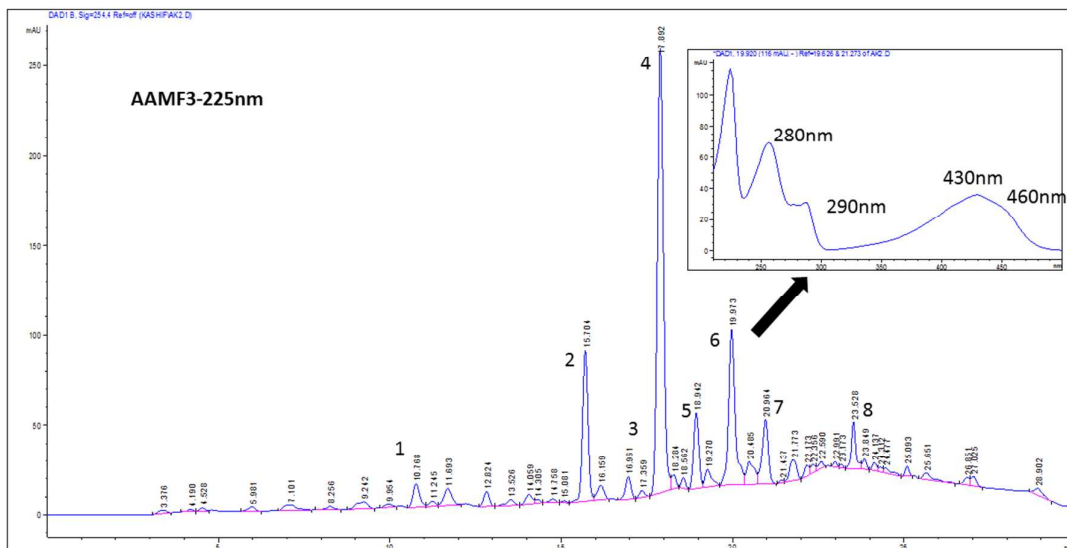


Figure S2. HPLC-PDA Chromatogram of AAMF3 of *Asparagus adscendens* extract monitored at 254nm.

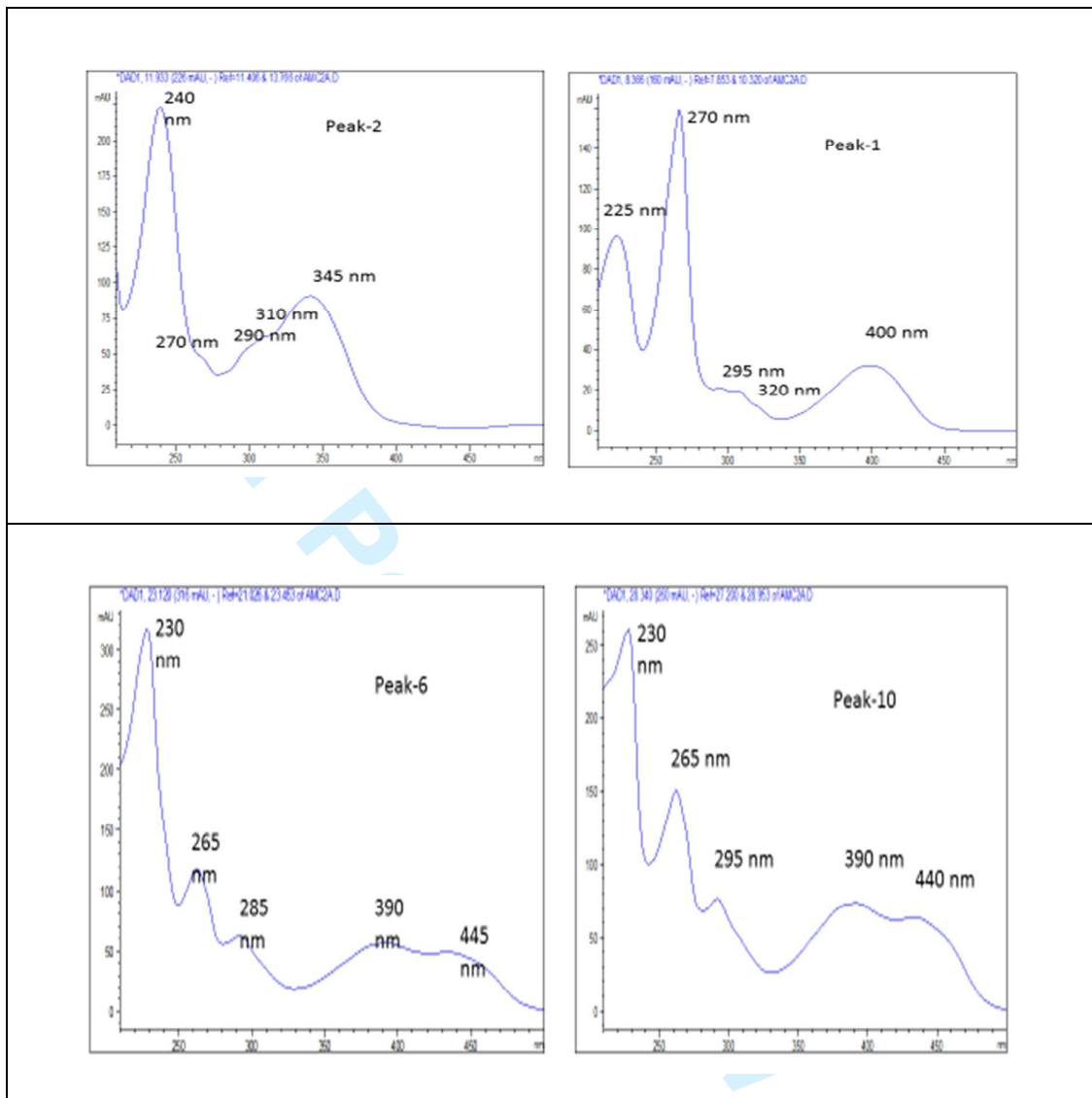


Figure S3: Corresponding UV-vis absorbance (AAMF1) at multiple wavelengths of the peaks separated by HPLC

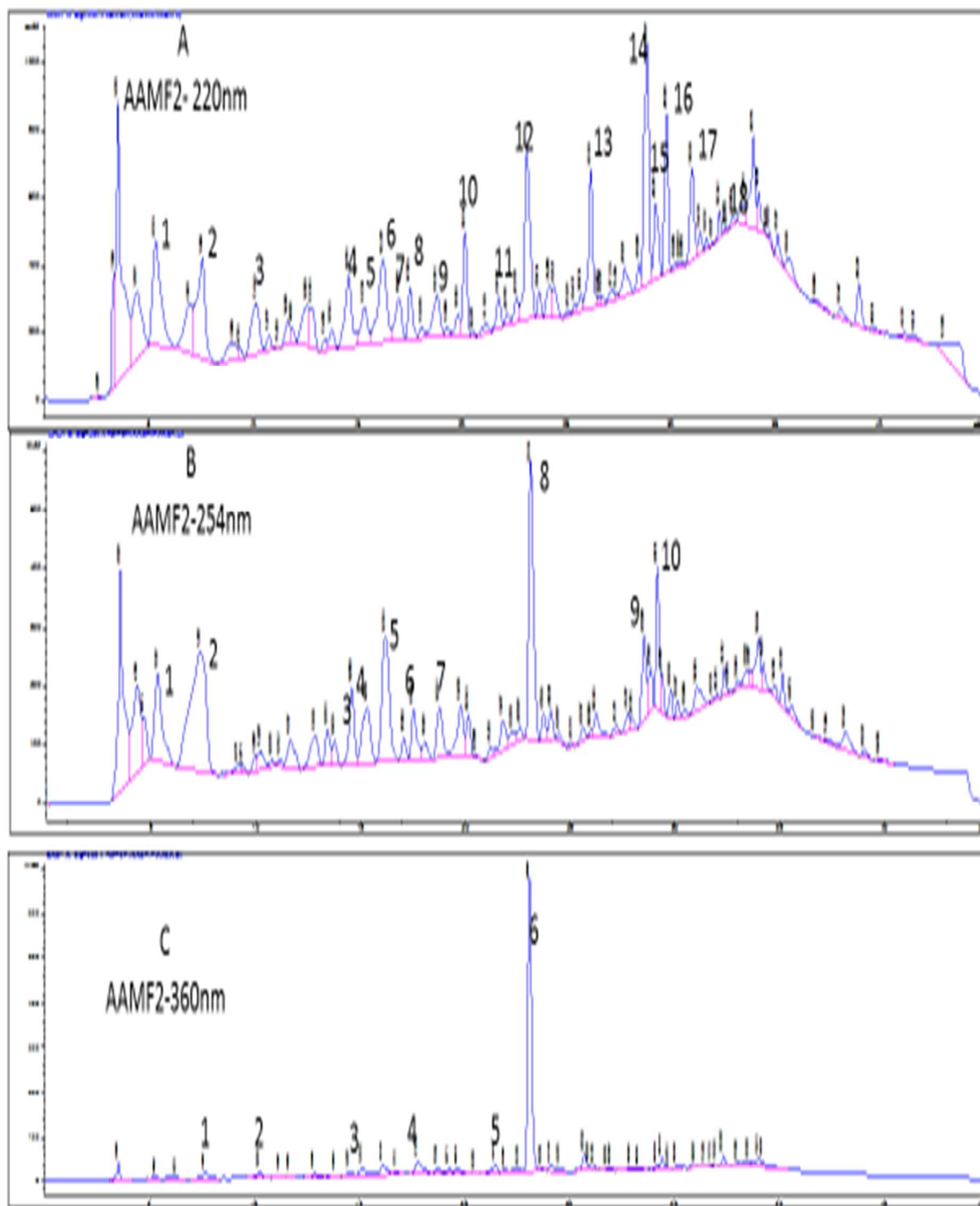


Figure S4: HPLC-PDA Chromatogram of AAMF2 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.

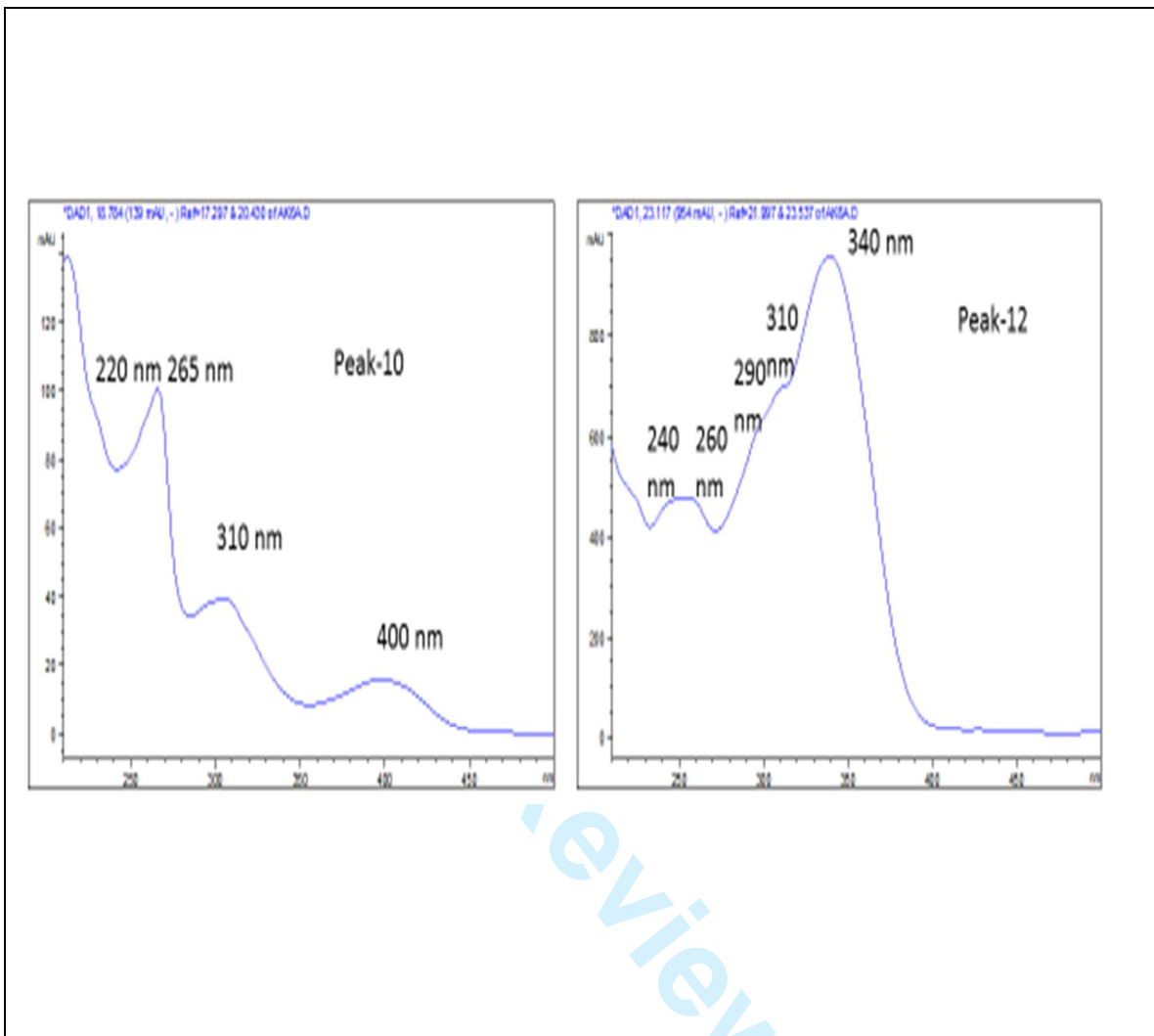


Figure S5: Corresponding UV-vis absorbance (AAMF2) at multiple wavelengths of the peaks separated by HPLC-PDA

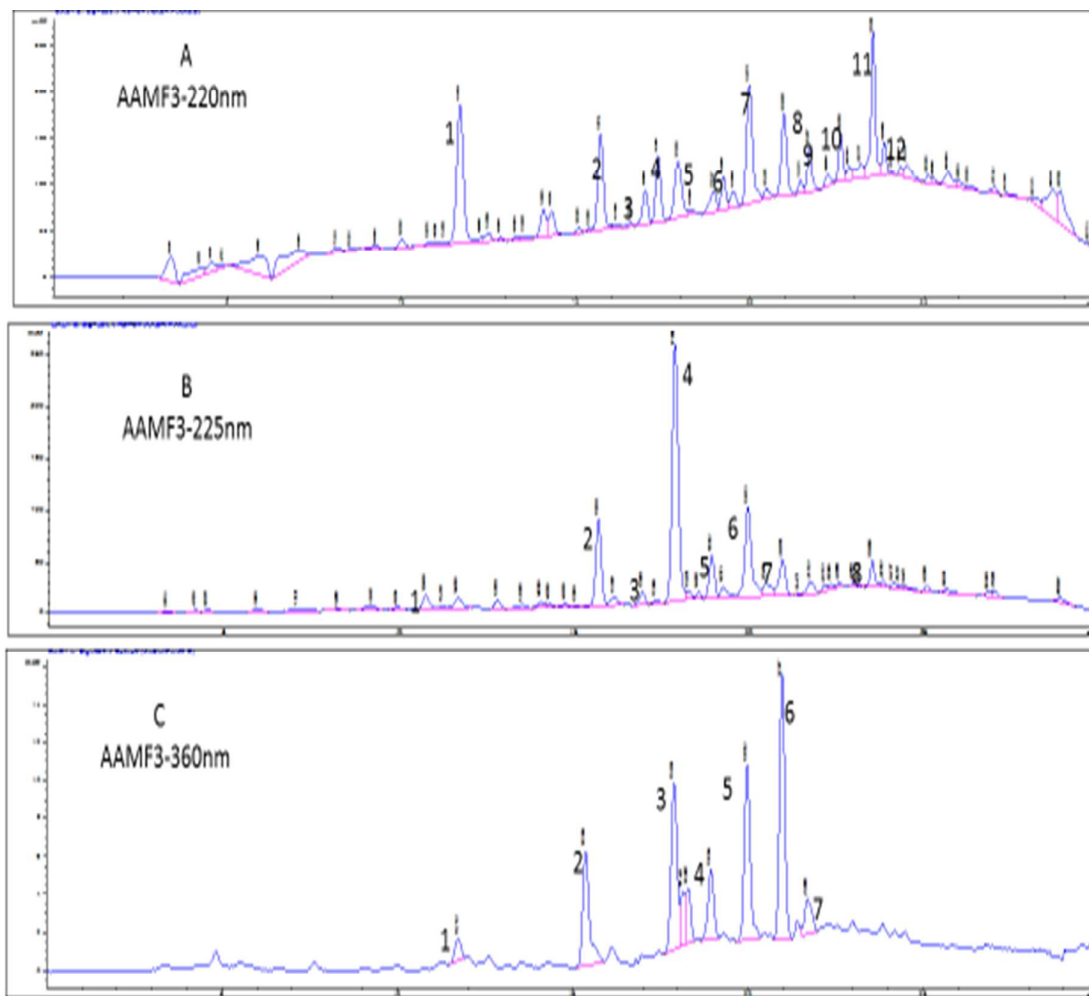


Figure S6: HPLC-PDA Chromatogram of AAMF3 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 225nm and (C) monitored at 360nm.

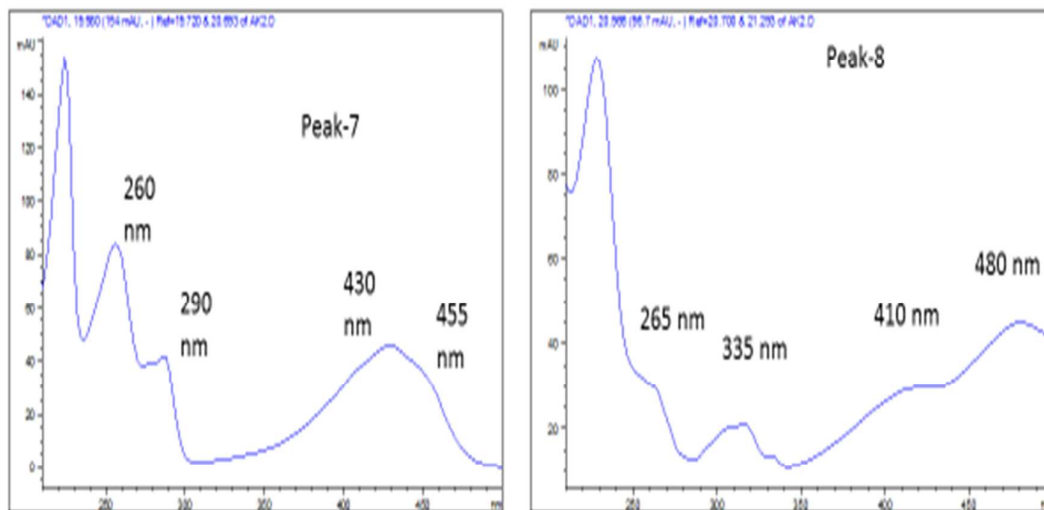


Figure S7: Corresponding UV-vis absorbance (AAMF3) at multiple wavelengths of the peaks separated by HPLC-PDA

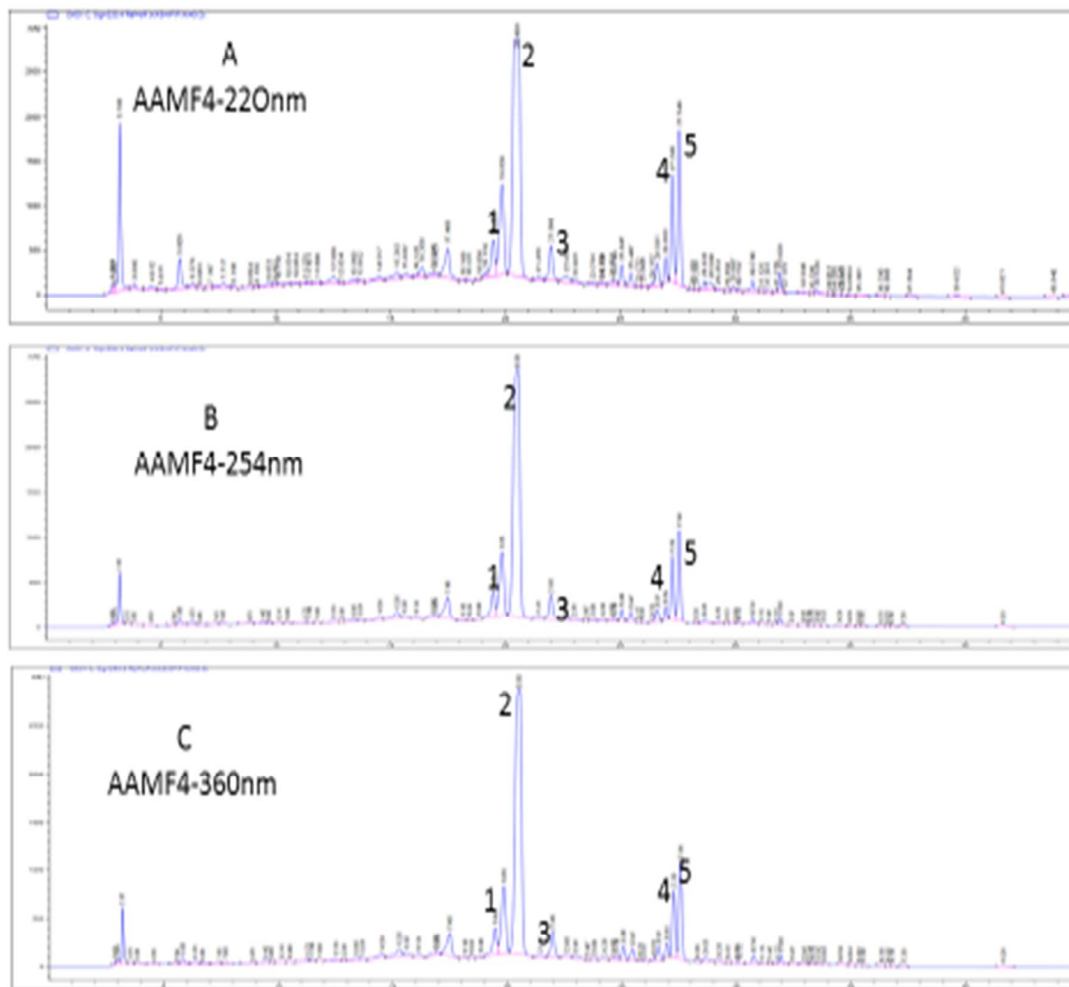


Figure S8: HPLC-PDA Chromatogram of AAMF4 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.

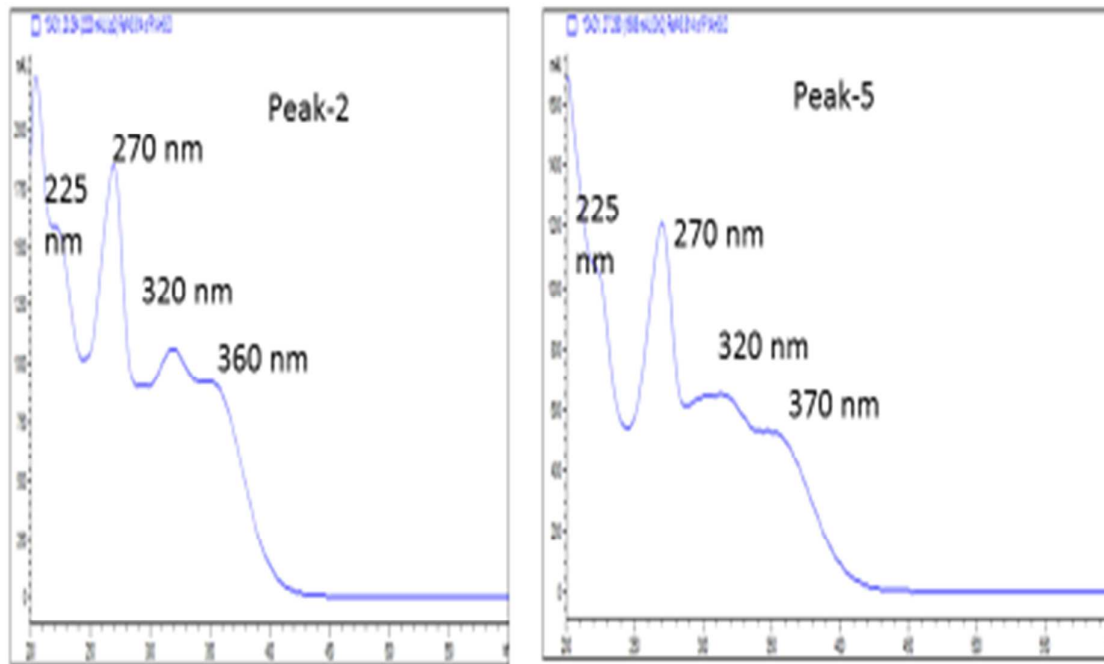


Figure S9: Corresponding UV-vis absorbance (AAMF4) at multiple wavelengths of the peaks separated by HPLC-PDA.

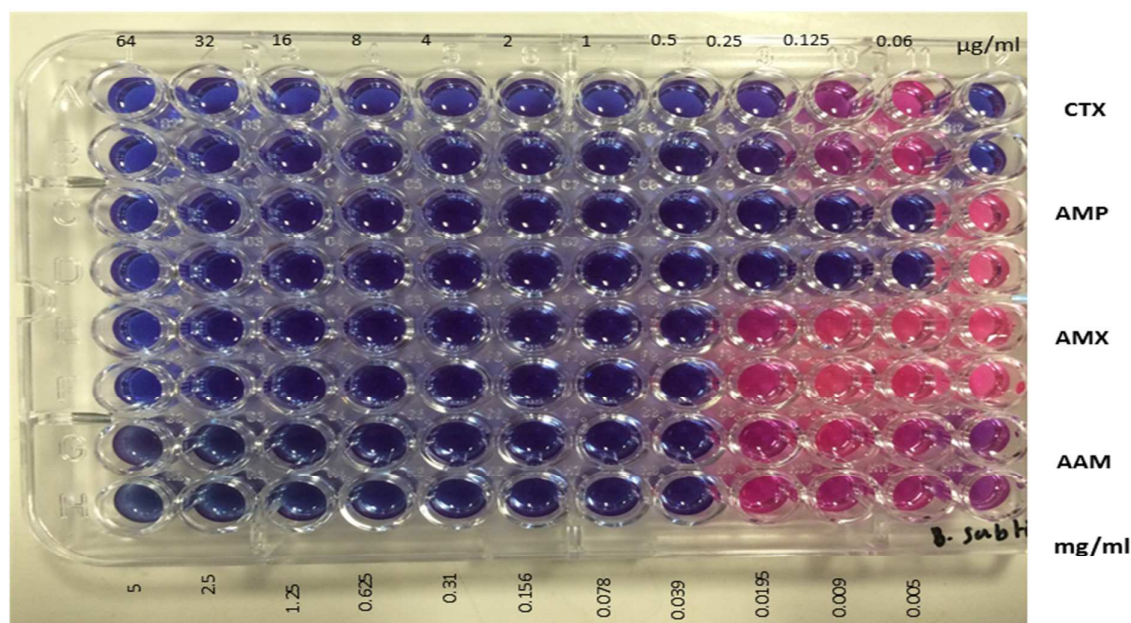


Figure S10: Typical Plate after 24 h in resazurin assay [final concentration of antibiotics and the extracts were 0.06-64 $\mu\text{g/ml}$ and 0.005-5 mg/ml , respectively; lowest concentration showing no colorimetric change from blue (resazurin) to pink (resorufin) was noted as the MIC; the test organism 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

Test compounds	Bacterial strains				
	SA	BS	ML	EC	KO
AAM ^a (mg/mL)	2.5	0.039	0.31	2.5	1.25
AAMF1 (mg/mL)	2.5	2.5	0.078	2.5	2.5
AAMF2 (mg/mL)	1.25	0.31	0.31	2.5	2.5
AAMF3 (mg/mL)	0.625	0.0195	0.078	2.5	1.25
AAMF4 (mg/mL)	0.156	0.009	0.156	≥10	≥10
CTX ^b (μg/mL)	2	0.25	0.25	≤0.06	≤0.06
AMP ^c (μg/mL)	0.125	≤0.06	0.125	4	2
AMX ^d (μg/mL)	4	0.5	4	2	2

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^a (*Asparagus adscendens* methanol extract), CTX^b (Cefotaxime), AMP^c (Ampicillin), AMX^d (Amoxicillin)

Table S2. Retention times (t_R) 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*

AMF1		220nm					254nm					360nm							
Peaks	t_R in (min)					Peaks	t_R in (min)					Peaks	t_R in (min)						
1 ^a	8.36	225	270	295	320	400	1	7.47	220	280			1 ^a	12.85	240	260	290	310	
2 ^a	11.93	240	270	290	310	345	2 ^a	8.42	225	270	320	400	2 ^a	16.66	285	325	360		
3	17.4	220	265				3 ^a	12.91	260	295	310	340	3 ^a	28.34	265	295	385	440	
4	20.53	230	290				4	26.44	240	290									
6 ^a	23.12	230	265	290	285	445	5 ^a	28.34	265	290	390	440							
10 ^a	28.34	230	265	295	390	440	6	32.76	230	260									
12	37.65	220	270				8 ^a	36.21	295	375	390	440							
AAMF2																			
10 ^a	18.78	220	230	265	310	400	2 ^a	8.42	225	270	320	400	1 ^a	12.85	240	260	290	310	
12 ^a	23.11	240	260	290	310	340	5 ^a	28.3	265	290	390	440	2 ^a	16.66	285	295	325	360	
13	26.16	220	260				8 ^a	36.2	295	375	390	440	3 ^a	28.34	265	295	385	440	
14	28.83	230	280																
AAMF3																			
1	11.68	225	275				2	15.72	220	245			2 ^a	15.39	225	245	260	335	
2	15.74	220	240				4	17.89	260	280	330		3 ^a	17.91	260	280	320		
7 ^a	19.95	260	290	430	455		6 ^a	19.92	280	290	430	460	5 ^a	19.92	255	290	430	460	
8 ^a	20.96	265	335	410	480		7 ^a	20.94	320	335	415	480	7	21.7	280				
AAMF4																			
2 ^a	20.29	225	270	295	320	360	2 ^a	20.29	227	270	295	322	2 ^a	20.29	226	270	324	370	
5 ^a	27.28	225	270	320	370		5 ^a	27.28	222	270	310	360	5 ^a	27.28	228	270	325	368	

^aPossible 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 gluoside). 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.