

## SUPPLEMENTARY MATERIAL

### **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

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 C<sub>18</sub> 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.

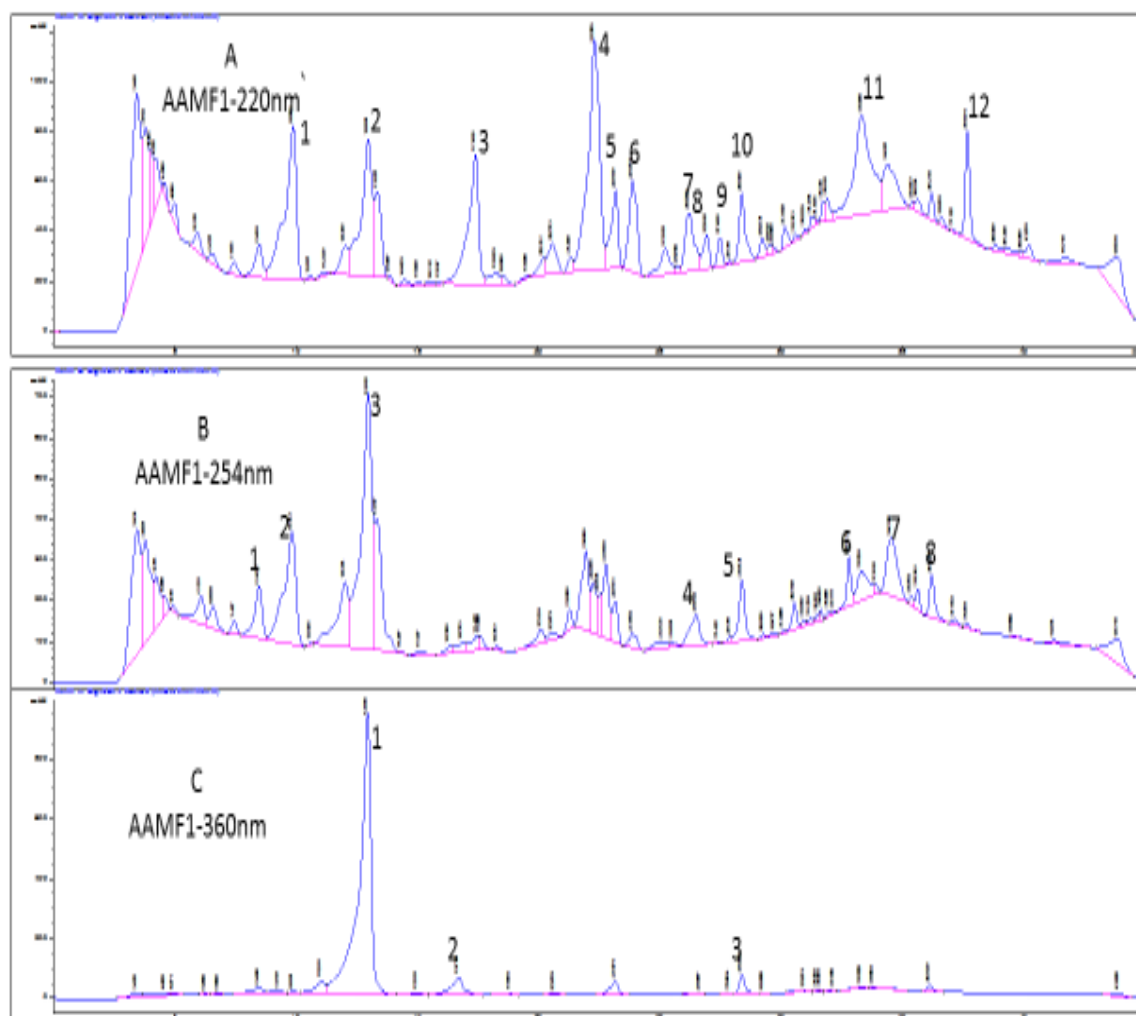
#### ***Minimum inhibitory concentration (MIC) determination***

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

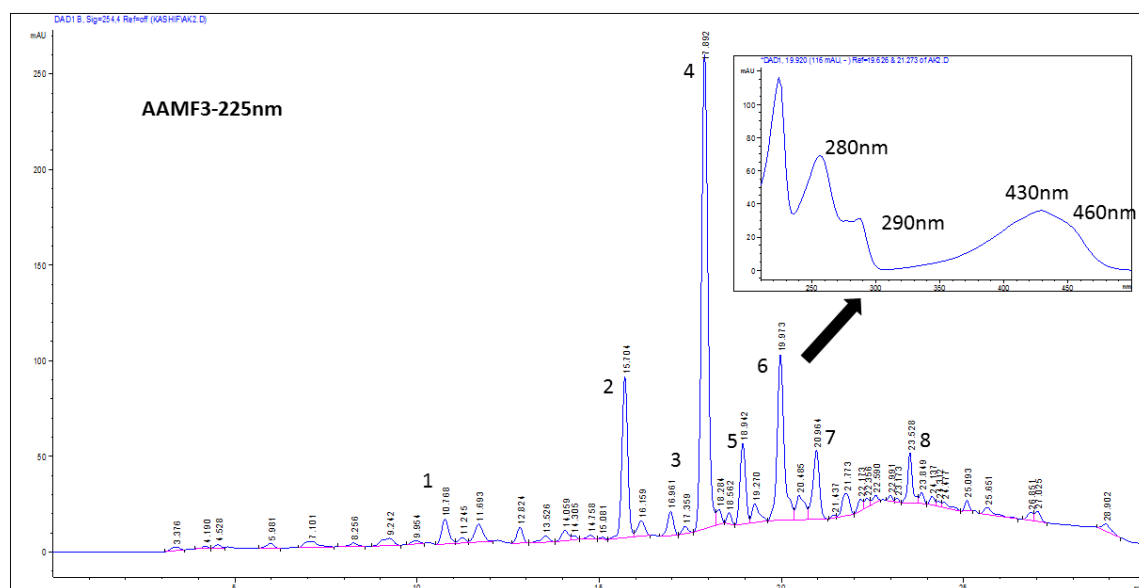
60  $\mu\text{L}$  of the CAMHB and 20  $\mu\text{L}$  of 0.02% resazurin were added to all wells. An Overnight culture of test bacteria was harvested by centrifugation at 4,000 rpm for 10 min, washed twice with NaCl by centrifugation at 4,000 rpm for 5 min each. The pellet collected was then adjusted approximately 0.5 standard McFarland equivalent (  $1 \times 10^8$  CFU/mL), diluted to give  $5 \times 10^6$  CFU/mL, and then 20  $\mu\text{L}$  will be transferred to the well so that the final concentration of inoculum is approximately  $5 \times 10^5$  CFU/mL. The total volume in each well was 200  $\mu\text{L}$  and the final concentration of antibiotics and the extracts were 0.06-64  $\mu\text{g/mL}$  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  $\pm$  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  $p \leq 0.05$  by using analysis of variance (ANOVA) with Tukey's range test.

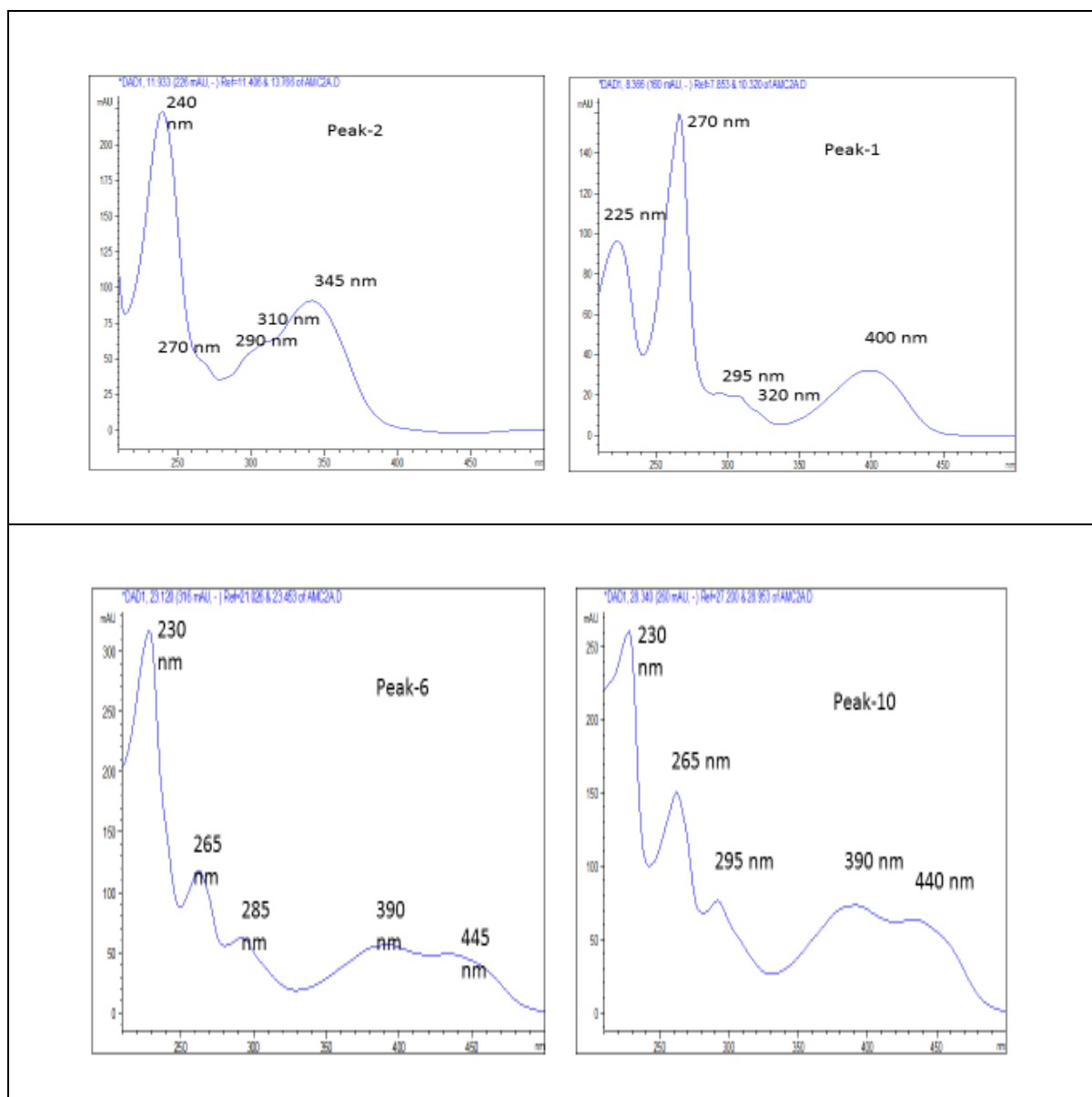
## **Results**



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

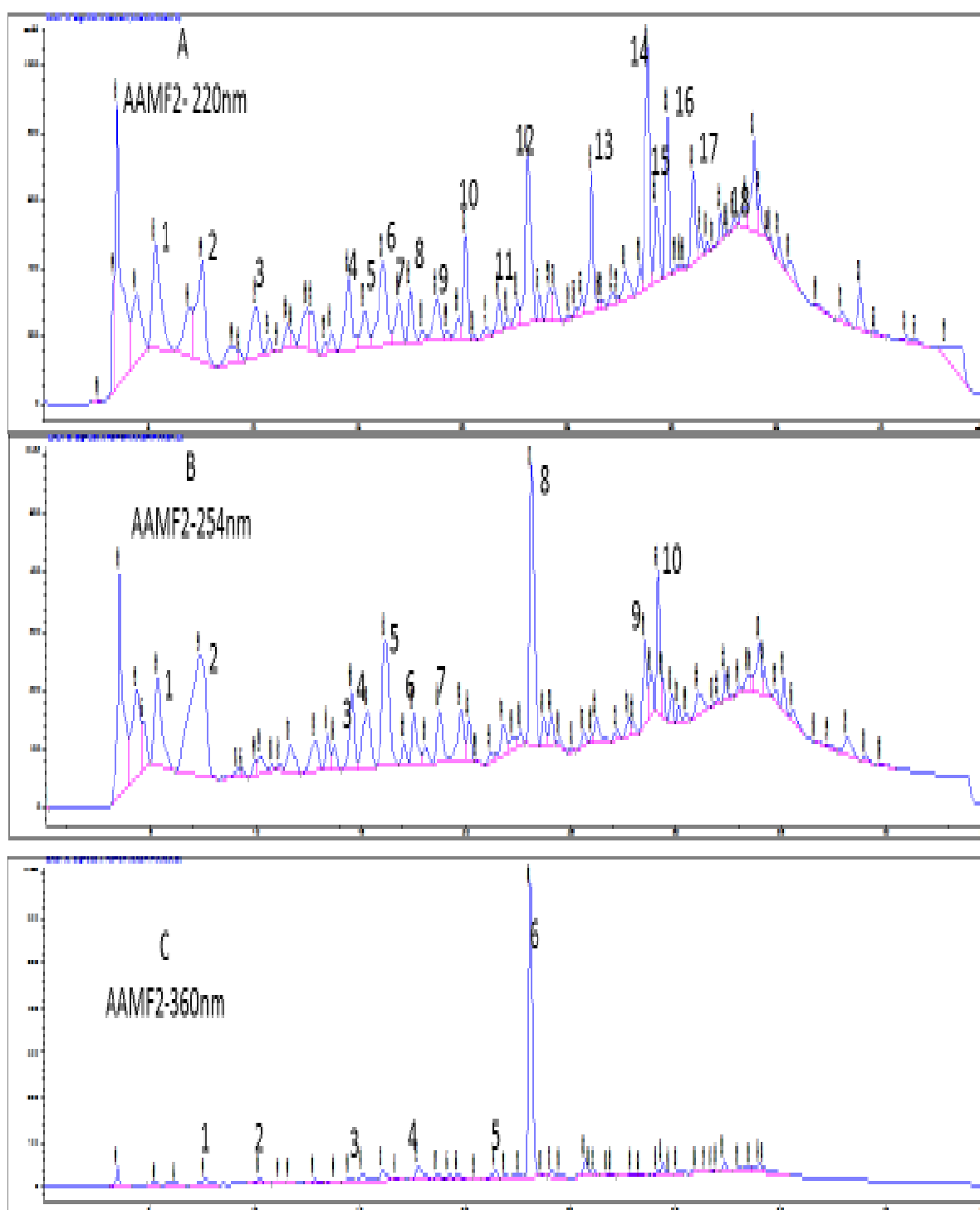


**Figure S2.** HPLC-PDA Chromatogram of AAMF3 of *Asparagus adscendens* extract monitored at 254nm.

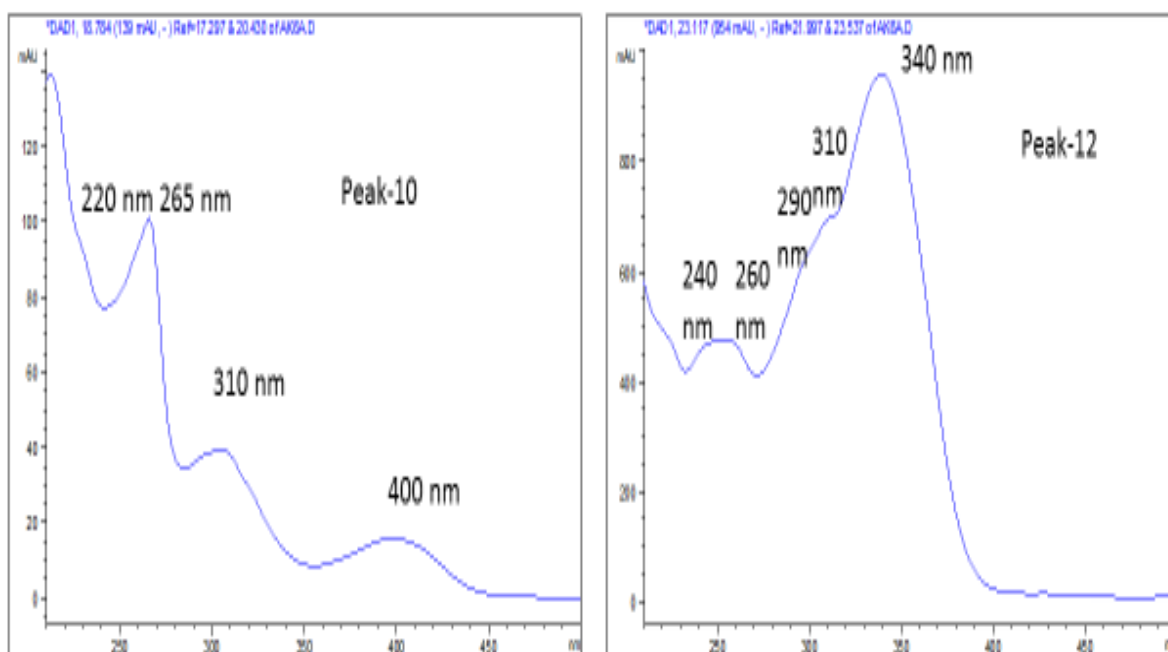


**Figure S3:** Corresponding UV-vis absorbance (AAMF1) at multiple wavelengths of the peaks separated by HPLC

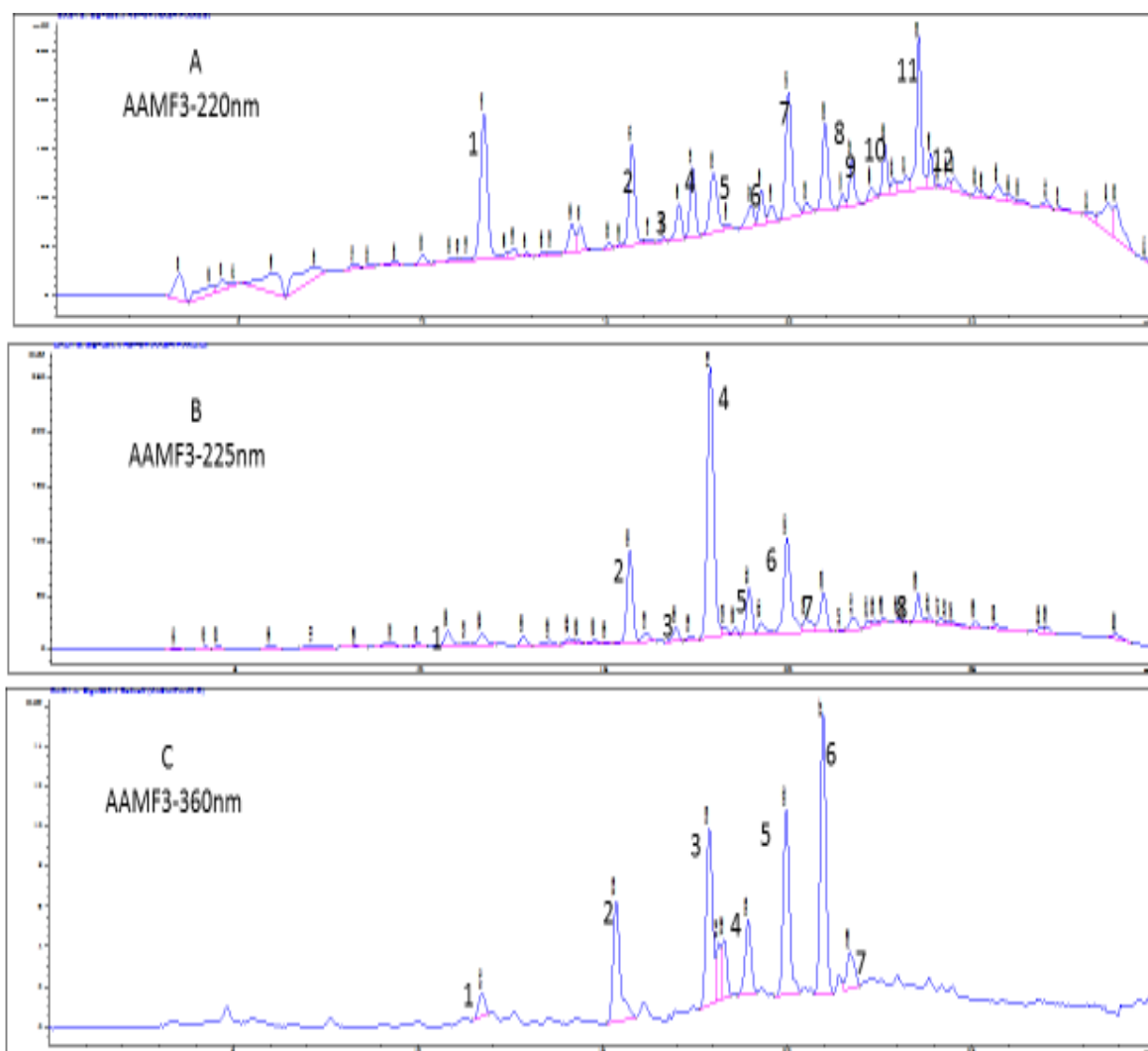




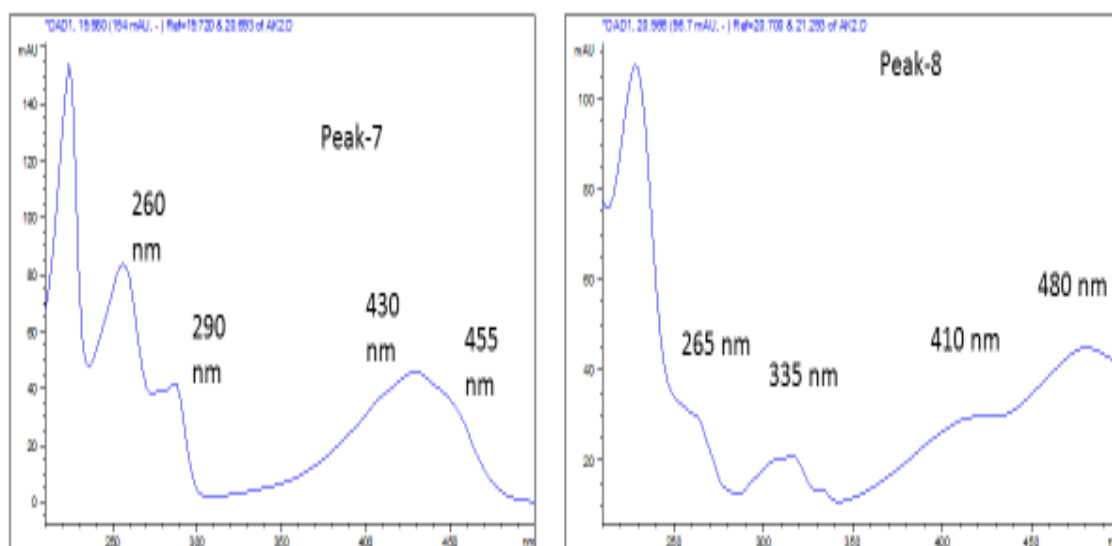
**Figure S4:** HPLC-PDA Chromatogram of AAMF2 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.



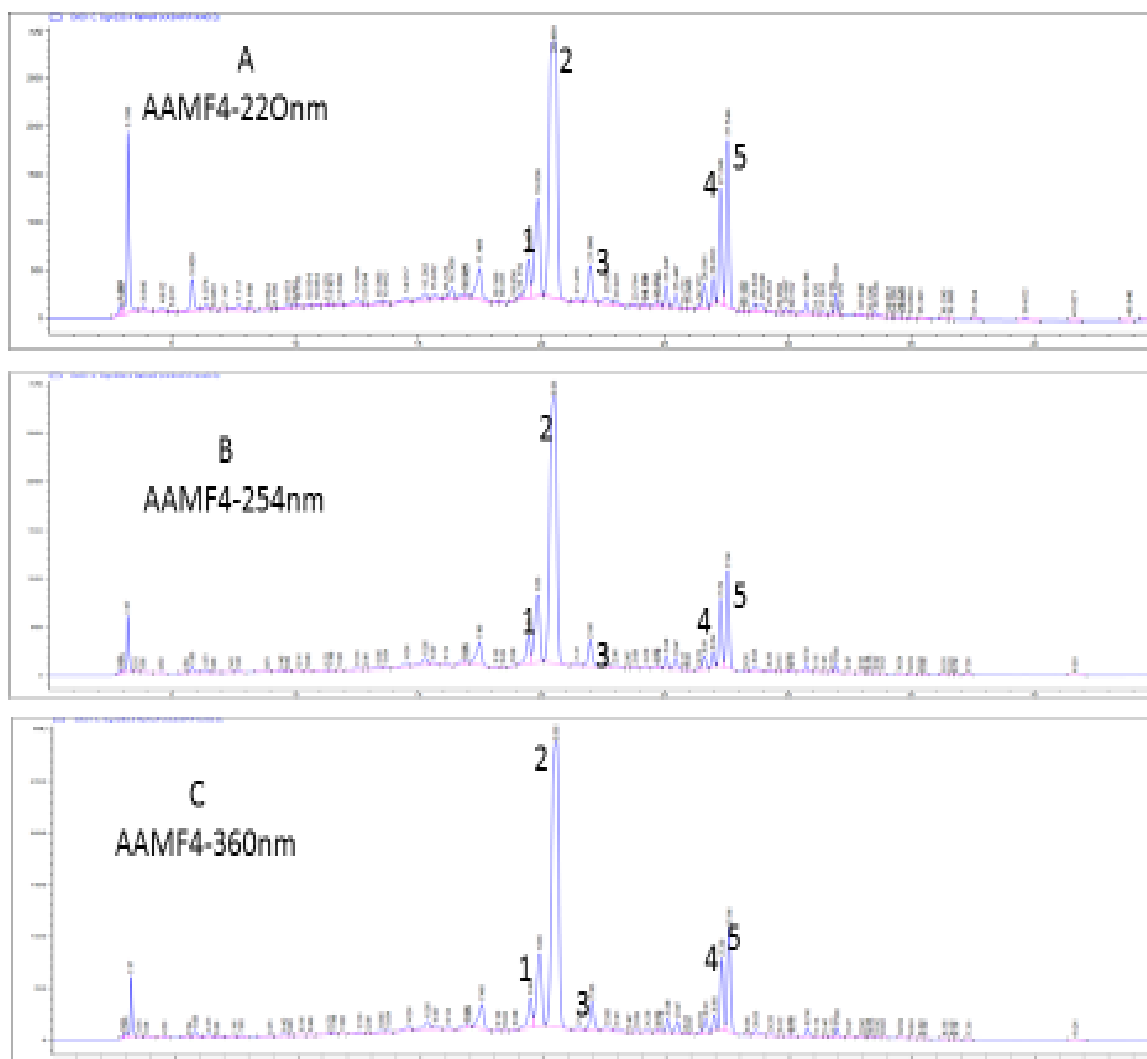
**Figure S5:** Corresponding UV-vis absorbance (AAMF2) at multiple wavelengths of the peaks separated by HPLC-PDA



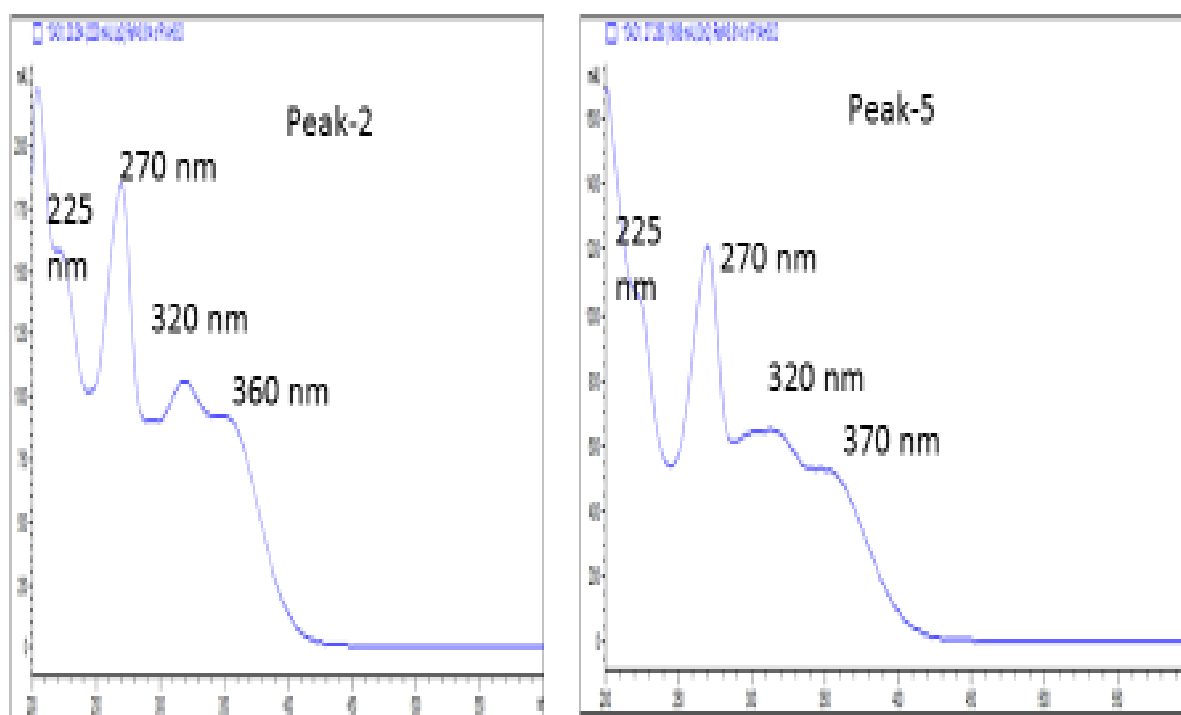
**Figure S6:** HPLC-PDA Chromatogram of AAMF3 of *Asparagus adscendens* extract (A) monitored at 220nm, (B) monitored at 254nm and (C) monitored at 360nm.



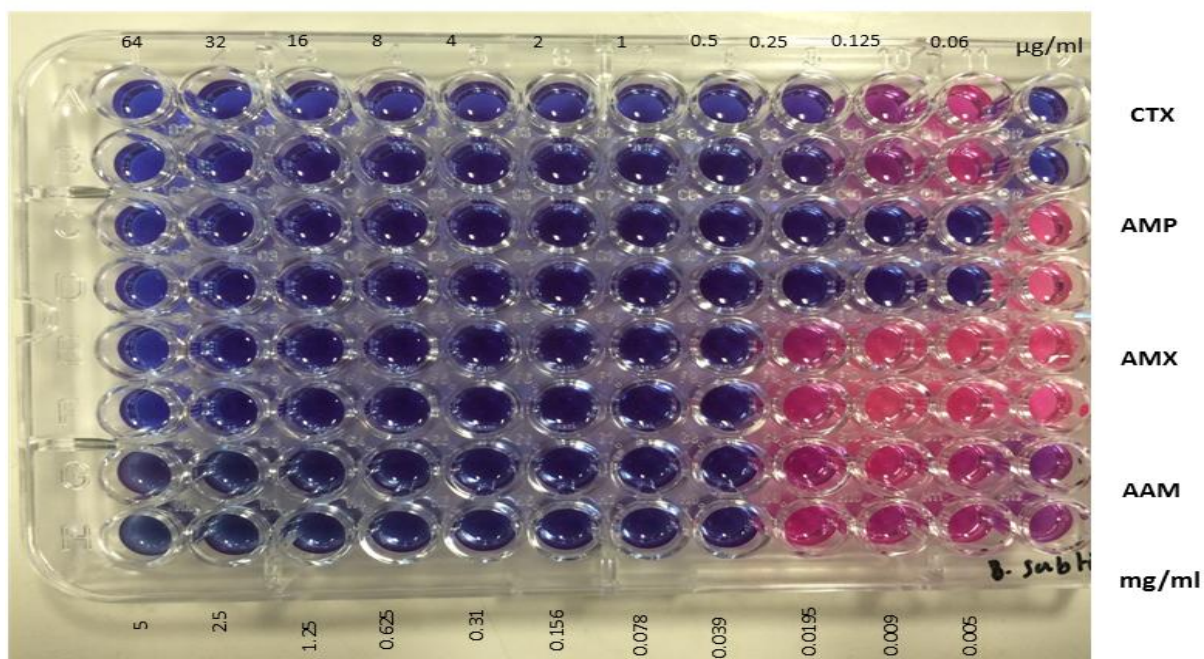
**Figure S7:** Corresponding UV-vis absorbance (AAMF3) at multiple wavelengths of the peaks separated by HPLC-PDA



**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  $\mu\text{g/ml}$  and 0.005-5  $\text{mg/ml}$ , 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		Bacterial strains				
compounds		SA	BS	ML	EC	KO
AAM <sup>a</sup>	(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 <sup>b</sup>	(μg/mL)	2	0.25	0.25	≤0.06	≤0.06
AMP <sup>c</sup>	(μg/mL)	0.125	≤0.06	0.125	4	2
AMX <sup>d</sup>	(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<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 ( $t_R$ ) 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 <sup>a</sup>	8.36	225	270	295	320	400	1	7.47	220	280				1 <sup>a</sup>	12.85	240	260	290	310
2 <sup>a</sup>	11.93	240	270	290	310	345	2 <sup>a</sup>	8.42	225	270	320	400		2 <sup>a</sup>	16.66	285	325	360	
3	17.4	220	265				3 <sup>a</sup>	12.91	260	295	310	340		3 <sup>a</sup>	28.34	265	295	385	440
4	20.53	230	290				4	26.44	240	290									
6 <sup>a</sup>	23.12	230	265	290	285	445	5 <sup>a</sup>	28.34	265	290	390	440							
10 <sup>a</sup>	28.34	230	265	295	390	440	6	32.76	230	260									
12	37.65	220	270				8 <sup>a</sup>	36.21	295	375	390	440							
<b>AAMF2</b>																			
10 <sup>a</sup>	18.78	220	230	265	310	400	2 <sup>a</sup>	8.42	225	270	320	400		1 <sup>a</sup>	12.85	240	260	290	310
12 <sup>a</sup>	23.11	240	260	290	310	340	5 <sup>a</sup>	28.3	265	290	390	440		2 <sup>a</sup>	16.66	285	295	325	360
13	26.16	220	260				8 <sup>a</sup>	36.2	295	375	390	440		3 <sup>a</sup>	28.34	265	295	385	440
14	28.83	230	280																
<b>AAMF3</b>																			
1	11.68	225	275				2	15.72	220	245				2 <sup>a</sup>	15.39	225	245	260	335
2	15.74	220	240				4	17.89	260	280	330			3 <sup>a</sup>	17.91	260	280	320	
7 <sup>a</sup>	19.95	260	290	430	455		6 <sup>a</sup>	19.92	280	290	430	460		5 <sup>a</sup>	19.92	255	290	430	460
8 <sup>a</sup>	20.96	265	335	410	480		7 <sup>a</sup>	20.94	320	335	415	480		7	21.7	280			
<b>AAMF4</b>																			
2 <sup>a</sup>	20.29	225	270	295	320	360	2 <sup>a</sup>	20.29	227	270	295	322		2 <sup>a</sup>	20.29	226	270	324	370
5 <sup>a</sup>	27.28	225	270	320	370		5 <sup>a</sup>	27.28	222	270	310	360		5 <sup>a</sup>	27.28	228	270	325	368

<sup>a</sup>Possible Phenolic Compounds