CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The heartwood of *Artocarpus lakoocha* was purchased from a traditional drugstore in Bangkok, and identified by comparison with authentic specimens in the Museum of Natural Medicine, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Puag-Haad was purchased from a traditional drugstore in Chiang Mai.

2. General Techniques

2.1. Thin-layer chromatography (TLC)

Technique	One dimension, ascending
Adsorbent	Silica gel GF_{254} (E. Merck) precoated plate (aluminium sheet) Aluminium oxide 60 F_{254} (E. Merck) neutral (type E) precoated plate (aluminium sheet)
Layer thickness	200 μm
Distance	5 cm, 8 cm
Temperature	Room temperature (30-35°C)
Detection	1. Ultraviolet light at wavelength of 254 and 365 nm 2. Anisaldehyde reagent and heat until colors developed.

2.2. Preparative thin-layer chromatography

Technique	One dimension, ascending
Adsorbent	Silica gel GF_{254} (E. Merck) precoated plate
Layer thickness	1 mm
Distance	18 cm.
Temperature	Room temperature (30-35°C)
Detection	Ultraviolet light at wavelength of 254 and 365 nm.

2.3 Vacuum liquid column chromatography

Adsorbent	Silica gel (No. 1.07734), particle size 0.063-0.200 mm. (70-230 mesh ASTM) (E. Merck) or Aluminium oxide (No. 1.01077), particle size 0.063-0.200 mm (70-230 mesh ASTM) (E. Merck) or Kieselguhr (Celite®)
Packing method	Dry packing with sintered glass filter column
Sample loading	The sample was dissolved in a small volume of organic solvent, mixed and triturated with a small quantity of adsorbent, dried and then applied gently on top of the column.
Detection	Separation was monitored by TLC observing under UV-light at 254 and 356 nm

2.4 Medium pressure liquid chromatography (MPLC)

Column packing	BÜCHI Cartridger™ C-670
Column	Polypropylene cartridges C-675 (40×150 mm) Porous HD-polyethylene frits
Packing Material	Silica gel (No. 1.07734), particle size 0.063-0.200 mm. (70-230 mesh ASTM) (E. Merck)
Flow rate	5 ml/min, 10 ml/min
Mobile phase	5% MeOH in CH_2Cl_2
Sample preparation	Crude Puag-Haad 2 g was dissolved in methanol (5 ml) and methylenechloride to make a 20 ml solution which was filtered through filter paper before injection.
Injection volume	20 ml
Pump	BÜCHI Pump Module C-605 BÜCHI Pump Manager C-615
Detector	BÜCHI UV Photometer C-635 at λ = 254 nm
Recorder	Liseis L 120 S

2.5 Spectroscopy

2.5.1 Ultraviolet (UV) absorption spectra

UV (MeOH and $CHCl_3$) spectra were obtained on a Milton Roy Spectronic 3000 Array Spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand).

2.5.2 Infrared (IR) absorption spectra

IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR 1760 Spectrophotometer with UATR technique (Chulabhorn Research Institute).

2.5.3 Mass spectra

Electron Spray Impact mass spectra (ESIMS) were measured on a Mass Finigan mat GCQ-Mass spectrometer (Chulabhorn Research Institute). Time-of-flight (TOF) mass spectra were measured with a Micromass mass spectrometer (BIOTEC).

High Resolution mass spectra were obtained in the Time-of-Flight technique (TOF) manner with a Bruker Daltonics mass spectrometer (Chulabhorn Research Institute) or a Micromass mass spectrometer (BIOTEC).

2.5.4 Proton and Carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained with a Bruker Avance DPX-300 FT-NMR spectrometer, (Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand). ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were obtained with a JEOL JNMA-500 FT-NMR spectrometer, (Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan).

Deuterated solvents for NMR spectra were $CDCl_3$, CD_3OD, DMSO-d_6, Acetone-d_6. Chemical shifts were reported in ppm scale using the solvent signal as reference.

2.6 Solvents

Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use.

3. Studies of methods for the isolation of oxyresveratrol

3.1 Extraction

Four methods of extraction were attempted and the weights of the extracts were compared. The amounts of oxyresveratrol in these extracts were then analyzed and compared using an HPLC method (see Section 5 for the analytical method).

3.1.1 The reflux method

The dried heartwood of *Artocarpus lakoocha* (120 g) was coarsely ground and refluxed with 600 ml of three different organic solvents (EtOAc, acetone or EtOH) for 8 hours. After filtration, the filtrate was dried under reduced pressure.

3.1.2 The soxhlet method

The dried heartwood of *Artocarpus lakoocha* (40 g) was coarsely ground and put in a soxhlet apparatus. Then 300 ml of an organic solvent (EtOAc, acetone or EtOH) was poured and the system was heated for 8 hours. After filtration, the filtrate was dried under reduced pressure.

3.1.3 The sonication method

The dried heartwood of *Artocarpus lakoocha* (40 g) was coarsely ground and sonicated in 300 ml of an organic solvent (EtOAc, acetone or EtOH) for 8 hours using an Elma® Transonic Digital (I = 5A, Hz-Frequency = 35 kHz, power level 9). After filtration, the filtrate was dried under reduced pressure.

3.1.4 The maceration method

3.1.4.(a) The dried heartwood of *Artocarpus lakoocha* (40 g) was coarsely ground and macerated with 300 ml of an organic solvent (EtOAc, acetone and EtOH) for 8 hours. After filtration, the filtrate was dried under reduced pressure.

3.1.4.(b) The dried heartwood of *Artocarpus lakoocha* 40 g was coarsely ground and macerated with 300 ml of an organic solvent (EtOAc, acetone or EtOH) for 2 days. After filtration, the filtrate was dried under reduced pressure.

All of the extracts obtained were analyzed for the quantity of oxyresveatrol by HPLC (see section 5)

Table 3: Weight of extracts

Method	Solvent	No.	Weight (g)	% oxyresveratrol based on weight of extract	% oxyresveratrol based on weight of plant material
1. Reflux	EtOH	1	4.1924	27.72	2.91
		2	4.2692	32.96	3.52
		3	3.6647	47.39	4.34
		Average (SD)	4.0421 (0.33)	36.02 (10.19)	3.59 (0.72)
	Acetone	1	2.2102	80.05	4.42
		2	2.4432	67.32	4.11
		3	2.0932	96.09	5.03
		Average (SD)	2.2489 (0.18)	81.15 (14.41)	4.52 (0.47)
	EtOAc	1	1.5206	89.91	3.42
		2	1.3795	89.68	3.09
		3	1.4090	90.55	3.19
		Average (SD)	1.4364 (0.07)	90.05 (0.45)	3.23 (0.17)
2. Soxhlet	EtOH	1	4.0732	50.38	5.13
		2	4.1050	43.17	4.43
		3	3.9866	67.09	6.69
		Average (SD)	4.0549 (0.06)	53.55 (12.27)	5.42 (1.16)
	Acetone	1	2.2657	64.10	3.63
		2	2.1279	76.02	4.04
		3	2.0598	71.90	3.70
		Average (SD)	2.1511 (0.10)	70.67 (6.05)	3.79 (0.22)

Method	Solvent	No.	Weight (g)	% oxyresveratrol based on weight of extract	% oxyresveratrol based on weight of plant material
	EtOAc	1	1.3742	48.64	1.67
		2	1.455	62.87	2.29
		3	1.2715	82.37	2.62
		Average (SD)	1.3669 (0.09)	64.63 (16.94)	2.19 (0.48)
3. Sonication	EtOH	1	2.2859	82.77	4.73
		2	2.2696	69.45	3.94
		3	2.1474	63.62	3.42
		Average (SD)	2.2343 (0.08)	71.95 (9.82)	4.03 (0.66)
	Acetone	1	0.9901	93.85	2.32
		2	0.9846	79.12	1.95
		3	0.9428	73.80	1.74
		Average (SD)	0.9725 (0.03)	82.26 (10.38)	2.00 (0.30)
	EtOAc	1	1.7918	94.45	4.23
		2	1.8355	90.83	4.17
		3	1.8416	93.82	4.32
		Average (SD)	1.8230 (0.03)	93.03 (1.94)	4.24 (0.08)
4 (a) Maceration (8 hr)	EtOH	1	2.0417	61.82	3.16
		2	1.8432	62.23	2.87
		3	1.7329	73.16	3.17
		Average	1.8726 (0.16)	65.74 (6.43)	3.07 (0.17)
	Acetone	1	1.5852	79.55	3.15
		2	1.7089	67.73	2.89
		3	1.4547	79.69	2.90
		Average	1.5829 (0.13)	75.66 (6.86)	2.98 (0.15)

Method	Solvent	No.	Weight (g)	% oxyresveratrol based on weight of extract	% oxyresveratrol based on weight of plant material
	EtOAc	1	0.9377	93.93	2.20
		2	0.9105	97.42	2.22
		3	0.9016	98.28	2.22
		Average (SD)	0.9166 (0.019)	96.54 (2.30)	2.21 (0.01)
4 (b) Maceration (48 hr)	EtOH	1	1.9768	61.27	3.03
		2	2.1544	70.98	3.82
		3	2.178	71.73	3.91
		Average (SD)	2.1031 (0.11)	67.99 (5.83)	3.59 (0.48)
	Acetone	1	1.8838	76.04	3.58
		2	2.0151	79.14	3.99
		3	2.3475	60.16	3.53
		Average (SD)	2.0821 (1.06)	71.78 (41.16)	3.70 (1.92)
	EtOAc	1	1.0735	96.25	2.58
		2	1.1326	87.58	2.48
		3	1.1821	103.72	3.07
		Average (SD)	1.1294 (0.05)	95.85 (8.08)	2.71 (0.31)

3.2 Separation

The dried heartwood of *Artocarpus lakoocha* (500 g) was coarsely ground and macerated with 6 L of ethanol for two days and filtered. The filtrate was evaporated under reduced pressure yielding brown syrupy mass (35.10 g). The EtOH extract prepared by the maceration method above was selected for separation studies due to its TLC/HPLC pattern showing separated oxyresveratrol spot on TLC and peak on HPLC chromatogram (Figure 5 and 6). Vacuum liquid chromatography was chosen for the separation experiment based on its ease to operate and the availability of the equipment in the laboratory. Three adsorbents in this experiment (17 g each) were silica gel (No. 1.07734, particle size 0.063-0.200 mm), aluminium oxide (Neutral/ No. 1.01077), particle

size 0.063-0.200 mm) and kieselguhr (Celite®). The adsorbent was packed in a 2.7 cm diameter sintered glass funnel. Then, the extract (1 g) was accurately weighed and triturated with small amount of adsorbent. The mixture was then placed gently on top of the column. Elution was performed with a gradient of EtOH/CH_2Cl_2 mixture (20 ml for each fraction), as shown in Table 4. The separation was monitored by examining the fractions on a TLC plate under UV-light (254 nm). The experiment was done in triplicate for each adsorbent. The results are shown in Table 5 and Figure 7.

Figure 5: The TLC chromatogram of oxyresveratrol (1); maceration with EtOH for 8 hrs (2); maceration with acetone for 8 hrs (3); maceration with EtOAC for 8 hrs (4); maceration with EtOH for 2 days (5); Puag-Haad (6). (from left spot to right spot)

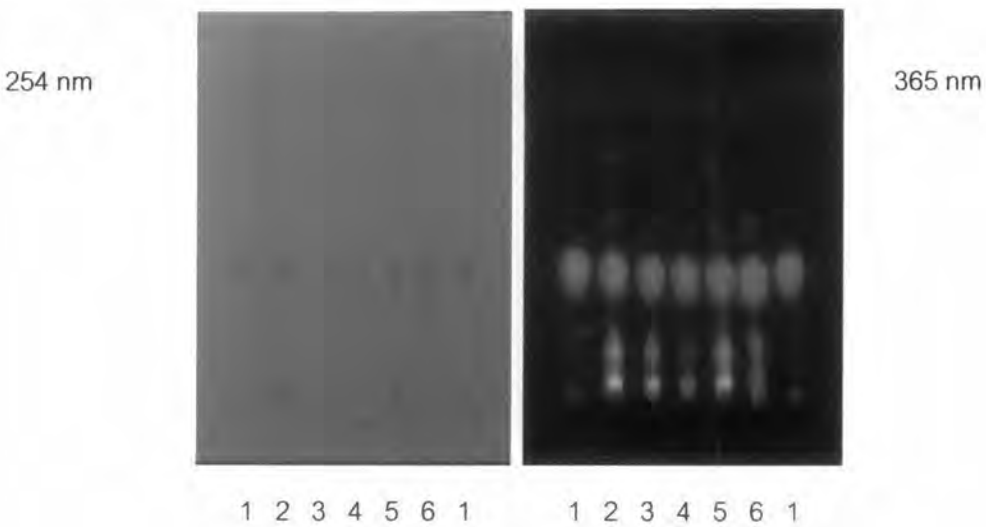


Table 4: Mixture of EtOH/CH_2Cl_2 for gradient elution

Fraction	%EtOH in CH_2Cl_2
1-3	5
4-8	10
9-13	15
14-18	20
19-22	30

Table 5: Fractions obtained with the adsorbent silica gel

Fraction	Weight (g) (from extract 1.0000 g)	Weight (g) (from extract 1.0001 g)	Weight (g) (from extract 1.0008 g)	Averaged weight (g) (mean)
1-3	0.0078	0.0130	0.0121	0.0110
4-9	0.1986	0.1803	0.1404	0.1731
10-14	0.2360	0.2856	0.2582	0.2599
15-18	0.1104	0.1066	0.1370	0.1180
19-22	0.1223	0.1176	0.1372	0.1257

Table 6: Fractions obtained with the adsorbent aluminium oxide

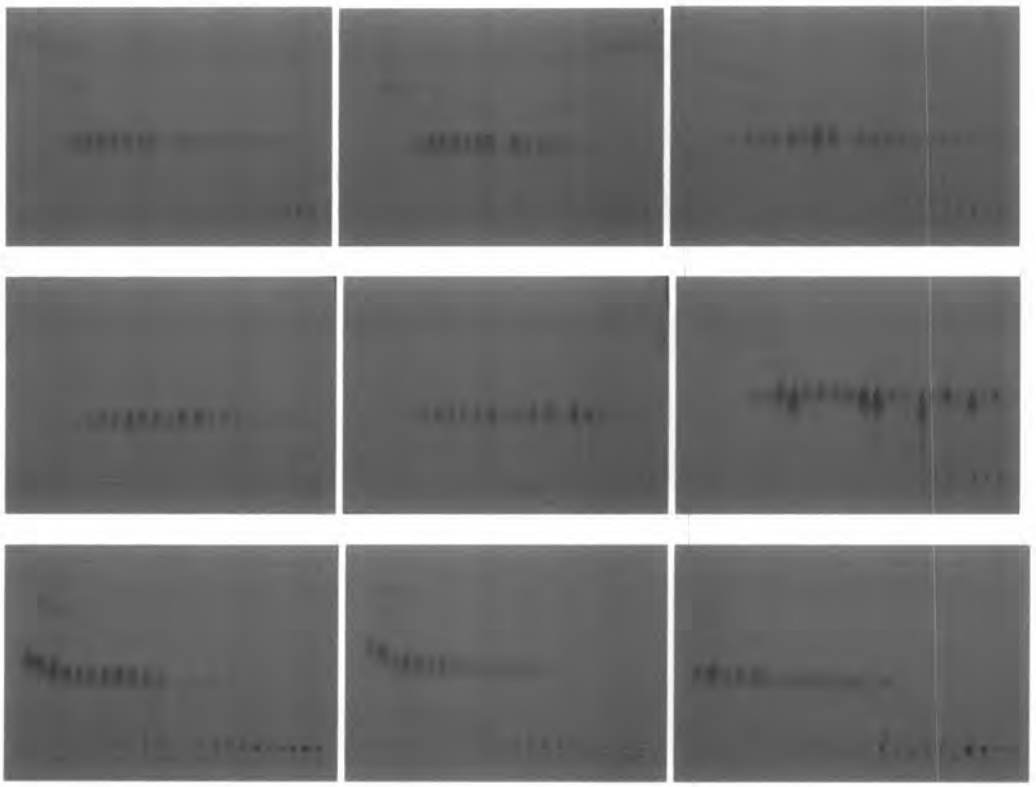
Fraction	Weight (g) (from extract 1.0004 g)	Weight (g) (from extract 1.0001 g)	Weight (g) (from extract 1.0005 g)	Averaged weight (g) (mean)
1-3	0.0085	0.0102	0.0141	0.0109
4-9	0.1023	0.0453	0.0527	0.0668
10-15	0.3407	0.3473	0.3756	0.3545
16-22	0.0636	0.1225	0.0763	0.0875

Table 7: Fractions obtained with the adsorbent kieselguhr

Fraction	Weight (g) (from extract 1.0008 g)	Weight (g) (from extract 1.0005 g)	Weight (g) (from extract 1.0003 g)	Averaged weigh (g) (mean)
1-3	0.0883	0.0951	0.1027	0.0954
4-9	0.4067	0.3800	0.3760	0.3876
10-18	0.2153	0.2042	0.2258	0.2151
19-22	0.1485	0.1716	0.1734	0.1645

Figure 6: TLC chromatogram of each fraction collected

From the first row - Silica gel column, the second row -Aluminium oxide and the third row -Kieselguhr as adsorbent (TLC solvent system 15% MeOH in CH_2Cl_2).



3.3 Isolation of Oxyresveratrol from Puag-Haad by MPLC

Crude Puag-Haad (2.0278 g) was dissolved in methanol (5 ml) and methylenechloride to make a 20 ml crude solution which was then injected into a packed column of MPLC. Elution was performed in an isocratic manner with 5% methanol in methylenechloride. Flow rate was adjusted to 5 ml/min for 30 minutes then to 10 ml/min till the end of extraction. Fractions were collected every 7 minutes. The first fraction was collected when the absorbance increased. Fractions with similar chromatographic pattern were combined to yield 1.0617 g (52.36% of the dry weight of Puag-Haad)

4. Method for quantitative analysis of oxyresveratrol

4.1 High performance liquid chromatography system

Although several reports on the isolation of oxyresveratrol from the heartwood of *A. lakoocha* have appeared (บุญชู ศรีตุลารักษ์ 2541; Venkataraman 1972; Mongolsuk *et al.*, 1957; Likhitwitayawuid, *et al*, 2005), none of them described a method for quantitative determination of the compound. In this study, a reversed-phase high performance liquid chromatography (HPLC) method is developed for analyzing oxyresveratrol in the extracts and the crude drug "Puag-haad," using the following conditions:

Column: Phenomenex hyperclone 5 µm C-18 150x4.5 mm with guard column

Flow rate: 0.3 ml/mim

Mobile phase: 10% H_2O in MeOH

Injection volume: 20 µl by manual injection

Pump: Shimadzu LC-10AD

Detector: Shimadzu SPD-10A λ = 254 nm

Integrator: Shimadzu C-RG8 Chromatopac

Statistical Analysis – software and graph Microsoft® Office Excel 2003

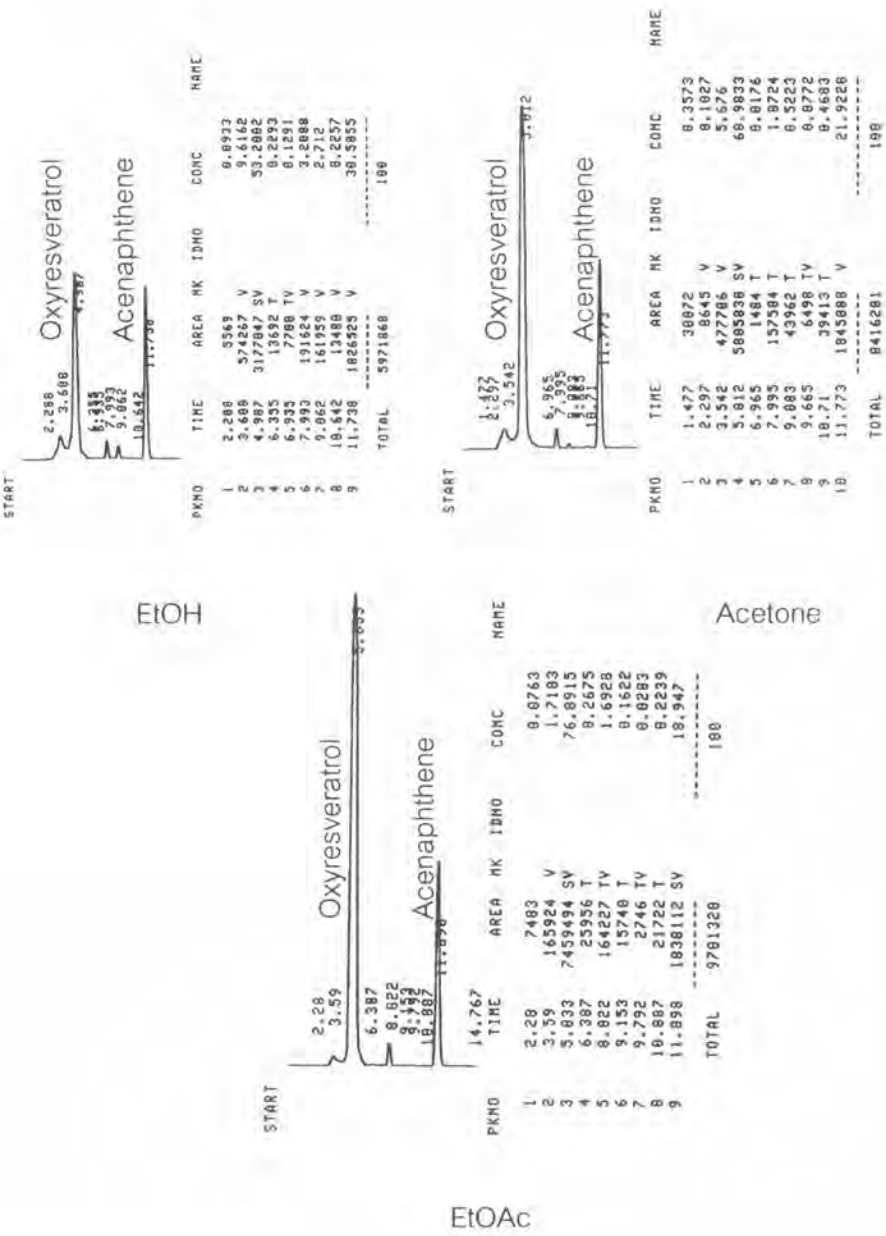
4.2 Internal standard

Several criteria were considered in finding a suitable internal standard (IS): (1) stability, (2) inability to interact with analyte, (3) detectability at interested wavelength (254 nm), and (4) resolution from the other components in the matrix sample. In this investigation, benzoic acid and acenaphthene were studied. Under the above HPLC condition, the former existed in two forms, i.e. ionized and unionized forms whereas the latter, reaching all of the afore-mentioned requirements, was selected for use as internal standard for the HPLC analysis.

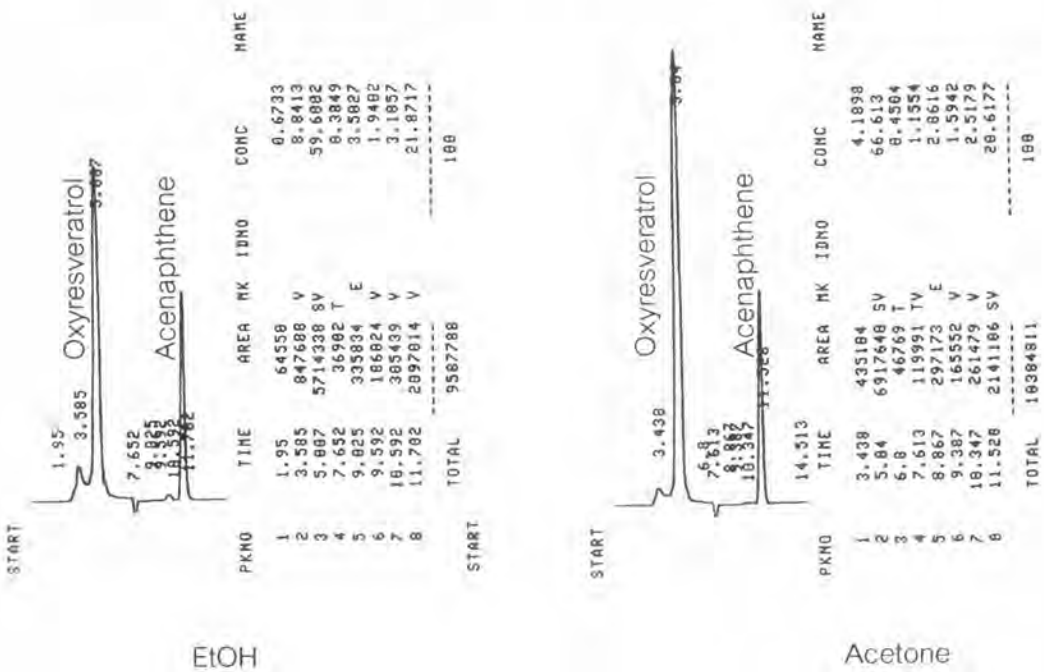
A solution of the internal standard was prepared by weighing accurately 100 mg of acenaphthene and dissolving it in MeOH. The volume of the obtained solution was then adjusted to 1000 ml by adding MeOH to give the final accurate concentration of 0.1 mg/ml of acenaphthene. This internal standard solution (IS) was used for all of the HPLC experiments. Figure 7 is the HPLC chromatogram showing peaks of oxyresveratrol at 5 minutes and acenaphthene at 11 minutes after injection.

Figure 7: The HPLC chromatograms

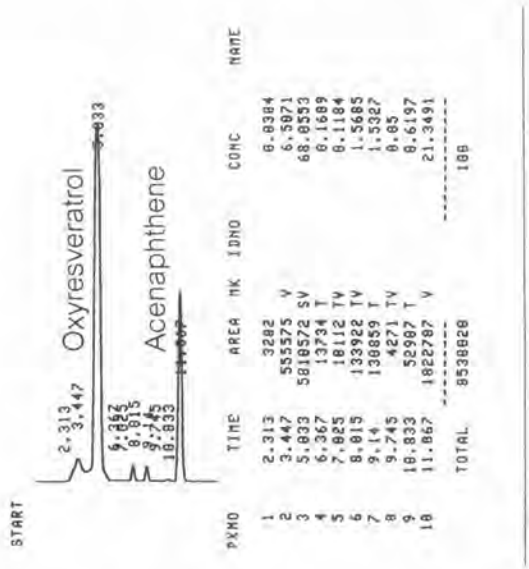
A: The HPLC chromatograms obtained from EtOH, acetone and EtOAc reflux



B: The HPLC chromatograms obtained from EtOH, acetone and EtOAc Soxhlet

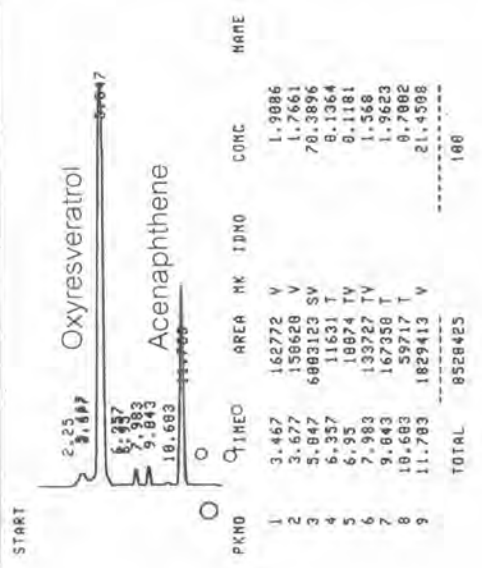


C: The HPLC chromatograms obtained from EtOH, acetone and EtOAc sonication



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.313	3282			0.0384	
2	3.447	555575	V		6.5071	
3	5.833	5818572	SV		68.0553	
4	6.367	13734	T		0.1609	
5	7.825	10112	TV		0.1184	
6	8.015	133922	TV		1.5685	
7	9.14	130859	T		1.5327	
8	9.745	4271	TV		0.05	
9	10.833	52907	T		0.6197	
10	11.867	1822787	V		21.3491	
TOTAL		8538828			100	

EtOH



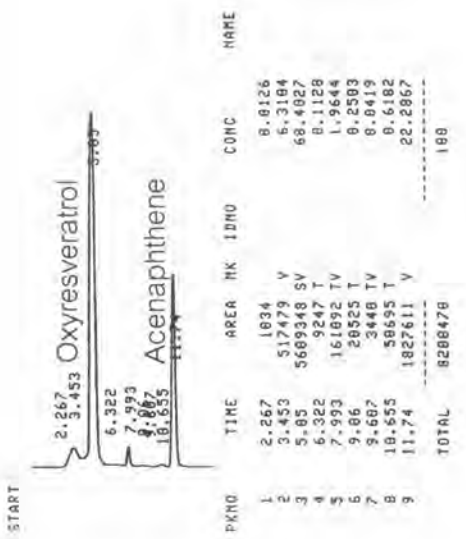
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.467	162772	V		1.9086	
2	3.677	156628	V		1.7661	
3	5.847	6083123	SV		70.3896	
4	6.357	11631	T		0.1364	
5	6.95	10074	TV		0.1181	
6	7.983	133727	TV		1.568	
7	9.843	167358	T		1.9623	
8	10.683	59717	T		0.7002	
9	11.783	1829413	V		21.4598	
TOTAL		8528425			100	

Acetone

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.413	139848			1.475	
2	5.065	6980813	SV		74.844	
3	6.368	13394	T		0.1421	
4	6.848	12504	TV		0.1326	
5	7.968	143360	TV		1.5208	
6	9.81	192834	T		2.0456	
7	10.522	119841	T		1.2713	
8	11.65	1825848	SV		19.3686	
TOTAL		9426838			100	

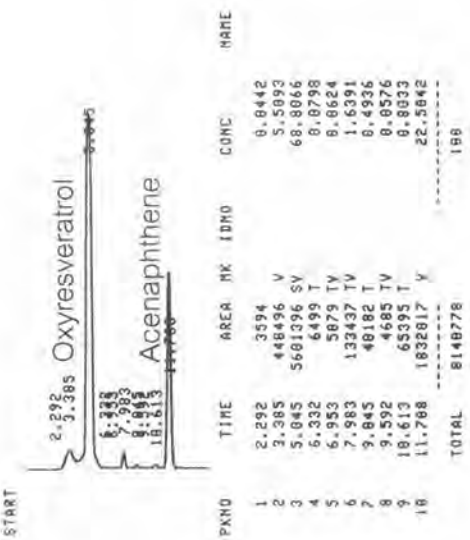
EtOAc

D: The HPLC chromatograms obtained from EtOH, acetone and EtOAc 8-hour maceration



PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.267	1034			0.0126	
2	3.453	517479	V		6.3194	
3	5.05	5609348	SV		68.4027	
4	6.322	9247	T		0.1128	
5	7.993	161092	TV		1.9644	
6	9.06	20525	T		0.2503	
7	9.607	3448	TV		0.0419	
8	10.555	50695	T		0.6182	
9	11.74	1827611	V		22.2867	
TOTAL		8200478			100	

EtOH



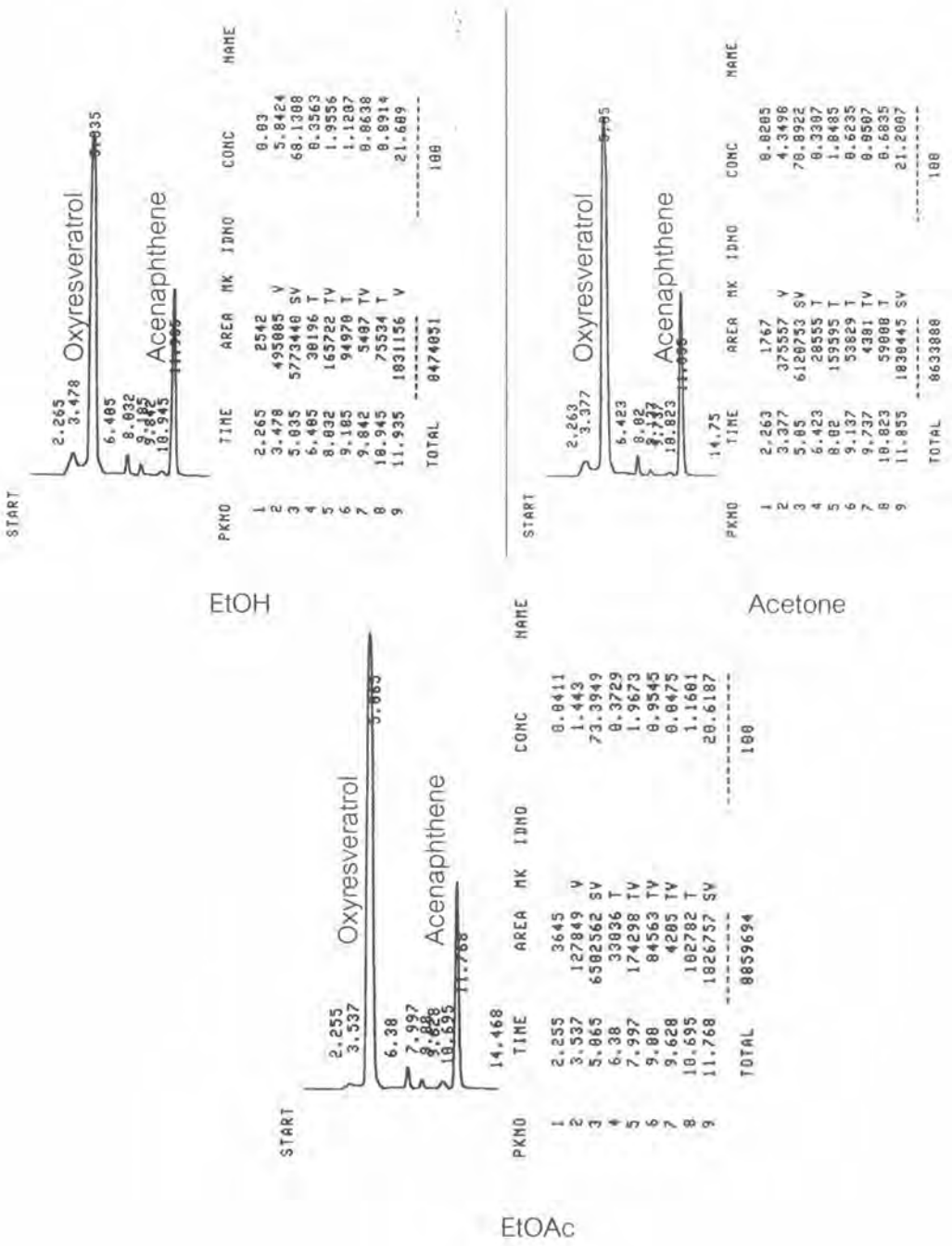
PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.292	3594			0.0442	
2	3.385	448496	V		5.5093	
3	5.045	5601396	SV		68.8066	
4	6.332	6499	T		0.0798	
5	6.953	5879	TV		0.0624	
6	7.983	133437	TV		1.6391	
7	9.845	40182	T		0.4936	
8	9.592	4685	TV		0.0576	
9	10.613	65395	T		0.8033	
10	11.788	1832817	V		22.5642	
TOTAL		8140778			100	

Acetone

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.342	2334			0.0247	
2	3.517	92475	V		0.9803	
3	5.053	7186297	SV		76.1796	
4	6.39	17985	T		0.1907	
5	7.817	11438	TV		0.1212	
6	8.818	149942	TV		1.5895	
7	9.15	17937	TV		0.1901	
8	9.817	5107	TV		0.0541	
9	10.877	182958	T		1.0871	
10	11.892	1847389	V		19.3822	
TOTAL		9433364			100	

EtOAc

E: The HPLC chromatograms obtained from EtOH, acetone and EtOAc 48-hour maceration



F: The HPLC chromatograms obtained from EtOAc 8-hour recovery experiment

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.56	48188			0.977	
2	5.88	2235141	SV		45.3155	
3	7	11285	T		0.2288	
4	7.68	92187	TV		1.869	
5	8.528	183217			3.7146	
6	9.387	142254	V		2.8841	
7	10.382	175465	V		3.5574	
8	11.577	2044658	SV		41.4537	
TOTAL		4932394			100	

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.217	13542			0.2216	
2	3.585	93608	V		1.5316	
3	5.092	3380466	SV		55.3118	
4	6.985	24234	T		0.3965	
5	7.698	74198	TV		1.214	
6	8.625	168482			2.7554	
7	9.532	158515	V		2.5937	
8	10.585	167273	V		2.737	
9	11.653	2031410	SV		33.2383	
TOTAL		6111647			100	

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.218	20571			0.2891	
2	3.598	64481	V		0.9062	
3	5.082	4401178	SV		61.8518	
4	6.983	23931	T		0.3363	
5	7.69	73446	TV		1.0322	
6	8.585	167318			2.3513	
7	9.512	152816	V		2.1476	
8	10.45	166156	V		2.3632	
9	11.617	2043798	SV		28.7224	
TOTAL		7115683			100	

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.225	23657			0.2987	
2	3.645	66863	V		0.8217	
3	4.118	43729	V		0.5374	
4	5.097	5389777	SV		66.2398	
5	6.965	17484	T		0.2149	
6	7.665	76555	TV		0.9409	
7	8.538	164816			2.0157	
8	9.272	135380	V		1.6638	
9	10.298	168744	V		2.0738	
10	11.512	2050556	SV		25.2011	
TOTAL		8136759			100	

4.3 Standard curve

Oxyresveratrol was accurately weighed (1 mg) and dissolved with the internal standard solution (10 ml) to give a standard solution of oxyresveratrol with an accurate concentration of 0.1 mg/ml (S1). Four additional standard solutions (S2, S3, S4 and S5) were then prepared from S1 by serial dilution. First, S2 were obtained by mixing 2 ml of S1 with 2 ml of IS. Then S3, S4 and S5 were prepared in a similar manner from S2, S3 and S4, respectively. Introduction of each standard solution to the HPLC was performed by a triplicate experiment. For each standard solution (S1 – S5), the area under curve (AUC) of oxyresveratrol (AUC S) and the AUC of acenaphthene (AUC IS) in each injection were measured and their ratio (AUC S/AUC IS) was determined. For each standard solution, an average value of the ratio (AUC S/AUC IS) was calculated based on the data from three injections (Table 8). A standard curve was then obtained by plotting (AUC S/AUC IS) ratios against the concentrations of oxyresveratrol. Figure 8a illustrates the standard oxyresveratrol curve with the equation $y = 22.509x + 0.3809$ and $R^2 = 0.9988$. An alternative way of obtaining a standard curve was by plotting the AUC of oxyresveratrol against the concentration with the equation $y = 5x10^7X + 623683$ and $R^2 = 0.998$, as shown in Figure 8b.

Table 8: Data of (AUC S), (AUC IS) and (AUC S/AUC IS) values of standard solutions

Standard	Conc. (mg/ml)	First injection			Second injection			Third injection			Average of AUC S/ AUC IS
		AUC S	AUC IS	AUC S/ AUC IS	AUC S	AUC IS	AUC S/ AUC IS	AUC S	AUC IS	AUC S/ AUC IS	
S 1	0.153	8153550	2085336	3.9099	8030045	2132718	3.7652	8090170	2161341	3.7431	3.8061
S 2	0.0765	4683031	2138965	2.1894	4597149	2209408	2.0807	4599604	2212754	2.0787	2.1163
S 3	0.03825	2416698	2073782	1.1654	2524130	1842214	1.3702	2557853	1878342	1.3618	1.2991
S 4	0.019125	1534732	1857953	0.8260	1534493	1855243	0.8271	1539019	1859482	0.8277	0.8269
S 5	0.0095625	1022505	1855380	0.5511	1021695	1857976	0.5499	1022765	2108465	0.4851	0.5287

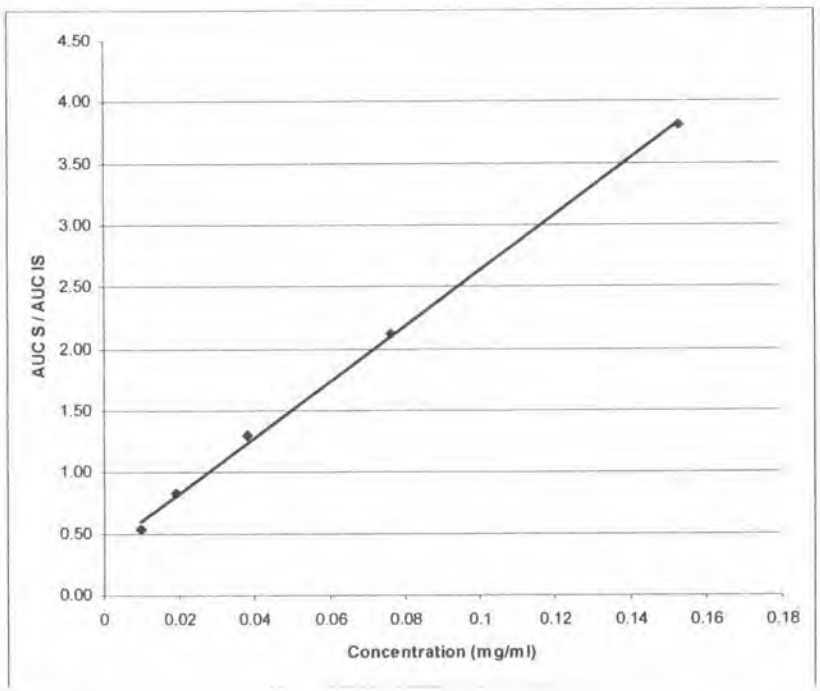


Figure 8a: Calibration curve of oxyresveratrol obtained from the AUC S / AUC IS ratio, equation: --- $y = 22.509x + 0.3809$

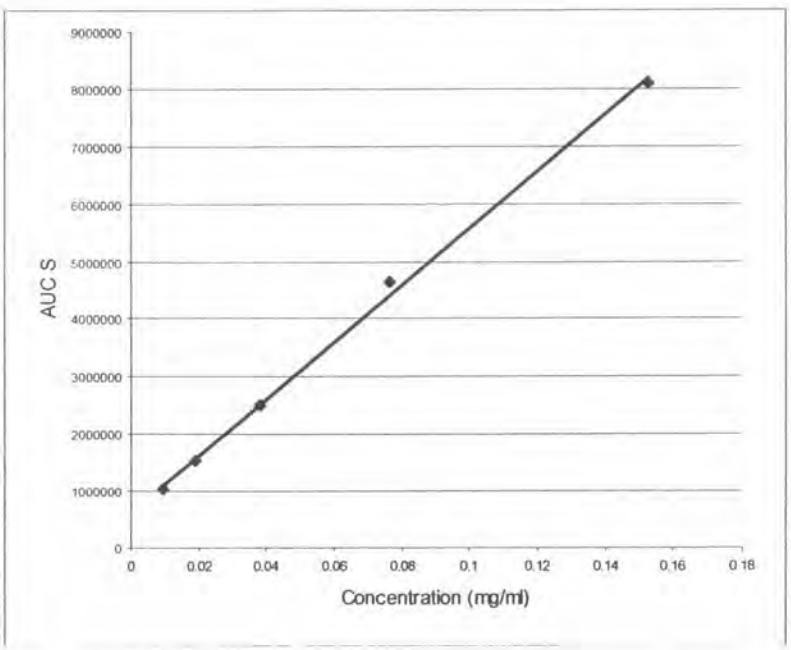


Figure 8b: Calibration curve of oxyresveratrol obtained from the AUC; equation --- $y = 5x10^{7}x + 623683$

4.4 Method validation

The newly developed HPLC method was subjected for validation on the following parameters of accuracy, precision, linearity, limit of detection (LOD) and limit of quantitation (LOQ).

4.4.1 Accuracy

Sample solutions (A_0, A_1, A_2 and A_3) for the determination of accuracy were prepared from an EtOAc extract (prepared by the 8-hour maceration). The crude extract was accurately weighed (4 mg) and dissolved in the internal standard solution (IS) (10 ml) to give sample A. Then the solution A (1 ml) was diluted in 10 ml volumetric flask to give A_0. Accurately weighed oxyresveratrol (10 mg) was dissolved in IS (25 ml) to give solution O_0. Then, 1 ml of O_0 was diluted with IS to the final volume of 10 ml to give a solution O_1. The concentration of oxyresveratrol in O_1 was determined using HPLC analysis (three 20 µl-injections) in connection with the standard curve representing the equation $y = 5x10^7x + 623683$. (Figure 8b), and the obtained value was used to calculate the quantity of oxyresveratrol in Solutions A_1, A_2 and A_3 (Table 9). Solution A_1 was prepared by mixing solution A_0 (1 ml) with O_0 (0.52 ml) (containing 0.20 mg oxyresveratrol) and the internal standard solution (IS) in a 10-ml volumetric flask. In a similar manner, solution A_0 was mixed with O_0 1.04 ml (containing 0.40 mg oxyresveratrol), and O_0 1.56 ml (containing 0.60 mg oxyresveratrol), which, after addition of appropriate amount of IS, gave solutions A_2, and A_3, respectively. Then, samples A_0, A_1, A_2 and A_3 (20 µl each) were subjected to HPLC analysis. Five injections were performed for each solution. The accuracy of the analytical method was expressed as percent recovery (%R) which was calculated according to the following equation. The results are shown in Table 10.

$$\%R = [(XE_i - XE_0) \times 100] / WS_i$$

Where XE_i = total analytical concentration of oxyresveratrol

XE_0 = analytical concentration of oxyresveratrol from extract solution (E_0)

WS_i = concentration of added oxyresveratrol

Table 9: Calculation of WS_i using the equation --- $y = 5x10^7x + 623683$

	Quantity of oxyresveratrol		
	O_0 (0.52 ml) in A_1	O_0 (1.04 ml) in A_2	O_0 (1.56 ml) in A_3
WS_i (mg/ml)	0.02	0.04	0.06

Table 10: Determination of accuracy

Injection Number	A_0		A_1		A_2		A_3	
	AUC	conc	AUC	conc	AUC	conc	AUC	conc
1	2283571	0.0332	3355906	0.0546	4360499	0.0747	5368298	0.0949
2	2236138	0.0322	3377593	0.0551	4330021	0.0741	5347737	0.0945
3	2235141	0.0322	3380466	0.0551	4401178	0.0755	5389777	0.0953
4	2239171	0.0323	3330758	0.0541	4332388	0.0742	5353325	0.0946
5	2297771	0.0335	3371390	0.0550	4310510	0.0737	5407101	0.0957
Average	2258358.4	0.0327	3363222.6	0.0548	4346919.2	0.0745	5373247.6	0.0950
Total analytical concentration (XE_i)				0.0548		0.0745		0.0950
Analytical concentration from the extract (XE_0)		0.0327		0.0327		0.0327		0.0327
Concentration of oxyresveratrol (mg/ml) (Analytical)				0.0639		0.0418		0.0623
Concentration of added oxyresveratrol (mg/ml) WS_i (Calculated)				0.02		0.04		0.06
%Recovery				110.5		104.5		103.8

4.4.3 Precision

Solution A_0 (20 µl) was injected into the HPLC system. The AUC was determined and the concentration of oxyresveratrol was calculated from the standard curve (Figure 8b). The mean concentration was then calculated from ten injections of the sample at the same time. The precision was expressed as percent relative standard deviation

(%RSD) or percent coefficient of variation (%CV), which was obtained from the standard deviation (SD), using the following equation.

%RSD or %CV = (SD / Mean) x 100

Where SD = standard deviation of analytical concentration

Mean = mean of analytical concentration

Table 11: Precision experiment

Injection number	Oxyresveratrol		Acenaphthene (internal standard)		Concentration (mg/ml)
	AUC	Concentration (mg/ml)	AUC	AUC/AUC IS	
1	2224711	0.0320	2049315	1.0856	0.0313
2	2283571	0.0332	2044007	1.1172	0.0327
3	2236138	0.0322	2059171	1.0859	0.0313
4	2217095	0.0319	2036482	1.0887	0.0314
5	2235141	0.0322	2044658	1.0932	0.0316
6	2239171	0.0323	2058570	1.0877	0.0314
7	2231109	0.0321	2059346	1.0834	0.0312
8	2297771	0.0335	2023701	1.1354	0.0335
9	2227146	0.0321	2061768	1.0802	0.0311
10	2218977	0.0319	2049238	1.0828	0.0312
Mean	2241083	0.03235	2048625.6	1.1039	0.0321
SD	27300.87	0.0005460	12038.69	0.0179	0.0008
% RSD	1.2182	1.6879	0.5876	1.6221	2.4767

4.4.4 Linearity

Five standard oxyresveratrol solutions with different concentrations were prepared by serial dilution as shown in Table 12. Each of the standard solution was injected into the HPLC system in triplicate. The averaged area under curve (AUC) was then calculated. A Plot of the AUC versus concentration was obtained. Linearity was expressed as a coefficient of determination (r^2).

Table 12: Linearity experiment

Concentration (mg/ml)	AUC of first injection	AUC of second injection	AUC of third injection	Average
0.0095625	1022505	1021695	1022765	1022321
0.019125	1534732	1534493	1539019	1536081
0.03825	2416698	2524130	2557853	2499560
0.0765	4683031	4597149	4599604	4626594
0.153	8153550	8030045	8090170	8091255
R^2				0.998

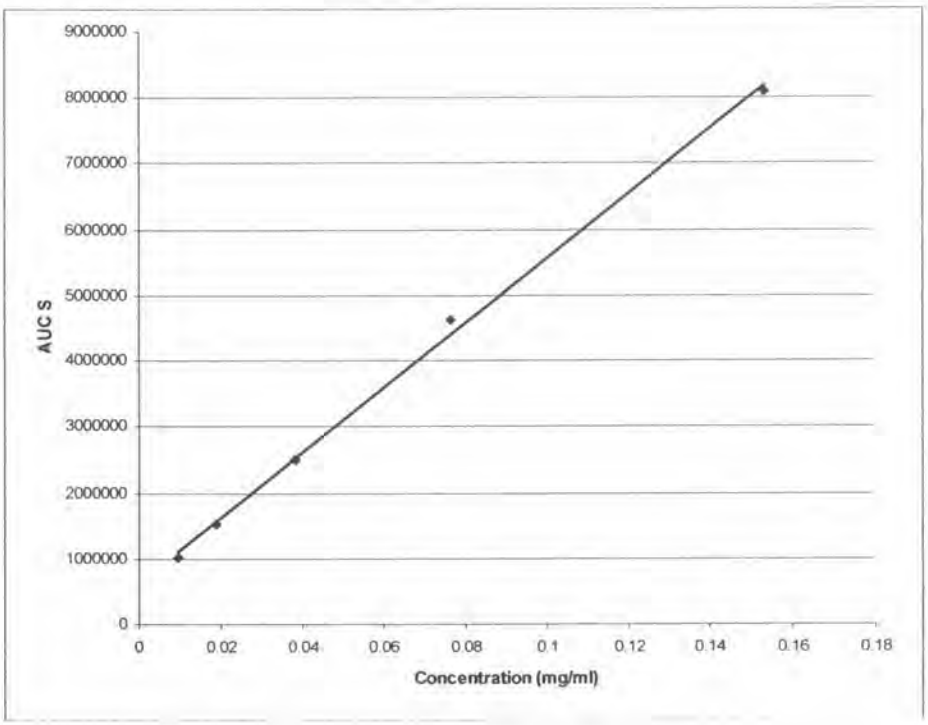


Figure 9: Linearity experiment

4.4.5 LOD and LOQ

Injection of each of the sample solutions (A_1, A_2 and A_3) to the HPLC system was carried out. A plot of mean concentrations versus standard deviations (SD) was then performed to reflect a linear correlation (Y = 0.0021X + 0.0004). A value of SD on the Y-axis intercept was obtained. The limit of detection (LOD) and limit of quantitation (LOQ) were then determined from the intercept value. The LOD is three times of Y-axis intercept, and the LOQ is ten times of Y-axis intercept. From the data below, the Y-axis intercept was 0.0004. Thus, LOD was 0.0012 mg/ml and LOQ was 0.004 mg/ml. (Taylor, 1989)

Table 13: Data of LOD and LOQ experiment

	AUC of A_1	Concentration mg/ml	AUC of A_2	Concentration mg/ml	AUC of A_3	Concentration mg/ml
1	3355906	0.054644	4360499	0.074736	5368298	0.094892
2	3377593	0.055078	4330021	0.074127	5347737	0.094481
3	3380466	0.055136	4401178	0.07555	5389777	0.095322
4	3330758	0.054142	4332388	0.074174	5353325	0.094593
5	3371390	0.054954	4310510	0.073737	5407101	0.095668
Average	3363223	0.054791	4346919	0.074465	5373248	0.094991
SD	20481.9	0.00041	35180.83	0.000704	24957.33	0.000499

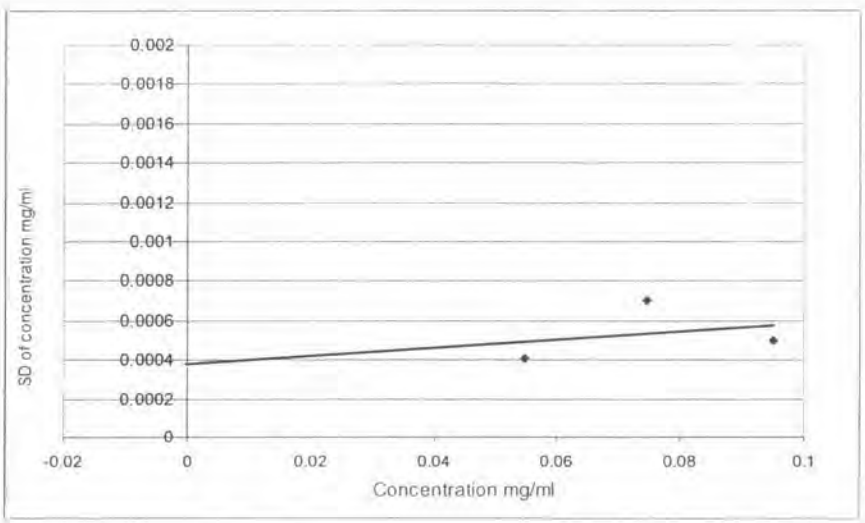


Figure 10: LOD and LOQ experiment

5. Quantitative analysis of oxyresveratrol in extracts and Puag-Haad

Acenaphthene was used as an internal standard for the HPLC analysis. A 0.1 mg/ml standard solution of acenaphthene was prepared by dissolving accurately weighed acenaphthene (100 mg) in 1000 ml of MeOH using a volume-metric flask.

Extracts were evaluated for their oxyresveratrol content by HPLC as described above. For each extract, the experiment was carried out in duplicate as follows. An accurately weighed extract was dissolved in the standard solution of acenaphthene. The mixture was then filtered through Millipore before subjecting to the HPLC injection. The mobile phase was 10% H_2O in MeOH on a C-18 column with 0.3 ml/min flow rate and UV detection at 254 nm. The AUCs of the oxyresveratrol and acenaphthene in the sample solution were summarized in Table 14 and the AUC/AUC IS ratios were illustrated in Table 15.

Table 14: The AUC data of extracted sample

	Experiment No.	Weight (mg)	First injection		Second injection	
			AUC	SI AUC	AUC	SI AUC
1. Reflux: EtOH	1	1.67	2582890	1824599	2614335	1828082
	2	1.84	3177047	1826525	3209376	1831152
	3	1.72	4071854	1845332	4047038	1819008
Acetone	1	1.72	6414533	1849517	6579056	1884239
	2	1.83	5805830	1845088	5844261	1848522
	3	1.73	7605452	1845207	7619750	1847696
EtOAc	1	1.9	7801241	1846943	7808061	1846578
	2	1.88	7637882	1837486	7723170	1840926
	3	1.8	7459494	1838112	7476661	1850047
2. Soxhlet: EtOH	1	2.03	5714338	2097014	5554282	2103558
	2	1.99	2411792	2108310	7415662	2127717
	3	1.93	7039929	2141565	7075554	2141565
Acetone	1	1.99	6917640	2141106	6976707	2131253
	2	1.98	7991918	2132557	7981900	2105986
	3	1.95	7511171	2109990	7459359	2123133
EtOAc	1	2.08	5726519	2158443	5759155	2162554
	2	1.75	6118912	2151287	6130124	2135534
	3	1.88	8193648	2129691	8364842	2152520

	Experiment No.	Weight (mg)	First injection		Second injection	
			AUC	SI AUC	AUC	SI AUC
3. Sonication: EtOH	1	1.92	7206638	1823000	7250546	1829651
	2	1.8	5810572	1822787	5859719	1830221
	3	1.57	4802815	1826105	4808528	1829609
Acetone	1	1.65	7048213	1831221	7224860	1860029
	2	1.64	6003123	1829413	6156841	1853583
	3	1.78	6087430	1824193	6109934	1830113
EtOAc	1	1.86	7981033	1845777	8008018	1842384
	2	1.66	6993424	1843163	6919843	1842798
	3	1.63	6980013	1825848	7018476	1835731
4. Maceration (8 hr): EtOH	1	1.89	5613877	1862941	5484964	1823362
	2	1.61	4757319	1848958	5163304	1913043
	3	1.63	5609348	1827611	5599431	1829297
Acetone 8 hrs RT	1	1.89	6890126	1835368	6937518	1837365
	2	1.76	5601396	1832017	5634384	1834766
	3	1.78	6499044	1824338	6576269	1834393
EtOAc 8 hrs RT	1	1.66	7186297	1847309	7201275	1850561
	2	1.79	7931251	1838835	7900302	1837824
	3	1.78	7949207	1836810	7958470	1846633
Maceration (48 hr): EtOH	1	2.01	5773440	1831156	5779334	1832900
	2	2	6551089	1830086	6543953	1831590
	3	1.93	6444384	1836908	6401265	1836529
Acetone 48 hrs RT	1	1.71	6120753	1830445	6002743	1834832
	2	1.68	6186170	1828764	6185369	1838601
	3	1.89	5400767	1826615	5360655	1833522
EtOAc 48 hrs RT	1	1.77	7675403	1814199	7614518	1812821
	2	1.62	6502562	1826757	6566253	1829362
	3	1.79	8345015	1837759	8410270	1836866
Puag-Haad	1	1.16	2227382	1865692	2241422	1866404
	2	1.01	2587396	2071900	2582451	2083996
	3	1.01	2519074	2059235	2503777	2078516

Table 15: The calculated data from the injection

		First injection	Second injection	Average	Concentration	% of oxyresveratrol	
		AUC /AUC IS	AUC /AUC IS		(mg/ml)	Based on weight of extract	Based on weight of heartwood
EtOH reflux	1	1.415593	1.430097	1.422845	0.04629		
	2	1.739394	1.752654	1.746024	0.060648	32.96	3.52
	3	2.20657	2.22486	2.215715	0.081515	47.39	4.34
Acetone reflux	1	3.468221	3.491625	3.479923	0.137679	80.05	4.42
	2	3.146641	3.161586	3.154114	0.123205	67.32	4.11
	3	4.121734	4.12392	4.122827	0.166241	96.09	5.03
EtOAc reflux	1	4.223867	4.228395	4.226131	0.170831	89.91	3.42
	2	4.156702	4.195264	4.175983	0.168603	89.68	3.09
	3	4.058237	4.041336	4.049786	0.162996	90.55	3.19
EtOH Soxhlet	1	2.724988	2.640423	2.682705	0.102262	50.38	5.13
	2	1.143946	3.485267	2.314606	0.085908	43.17	4.43
	3	3.287282	3.303917	3.2956	0.12949	67.09	6.69
Acetone Soxhlet	1	3.230872	3.273524	3.252198	0.127562	64.10	3.63
	2	3.747575	3.790101	3.768838	0.150515	76.02	4.04
	3	3.559814	3.513373	3.536593	0.140197	71.90	3.70
EtOAc Soxhlet	1	2.653079	2.663127	2.658103	0.101169	48.64	1.67
	2	2.844303	2.870534	2.857419	0.110023	62.87	2.29
	3	3.847341	3.886069	3.866705	0.154863	82.37	2.62
EtOH Sonicate	1	3.953175	3.962803	3.957989	0.158918	82.77	4.73
	2	3.187741	3.201646	3.194693	0.125007	69.45	3.94
	3	2.630087	2.628172	2.62913	0.099881	63.62	3.42
Acetone Sonicate	1	3.848914	3.884273	3.866594	0.154858	93.85	2.32
	2	3.281448	3.321589	3.301518	0.129753	79.12	1.95
	3	3.337054	3.338556	3.337805	0.131365	73.80	1.74
EtOAc Sonicate	1	4.323942	4.346552	4.335247	0.175678	94.45	4.23
	2	3.794252	3.755074	3.774663	0.150774	90.83	4.17
	3	3.822888	3.82326	3.823074	0.152924	93.82	4.32
EtOH 8 hrs RT	1	3.013449	3.00816	3.010804	0.116838	61.82	3.16
	2	2.572973	2.699	2.635987	0.100186	62.23	2.87
	3	3.069224	3.060974	3.065099	0.11925	73.16	3.17

		First injection	Second injection	Average	Concentration (mg/ml)	% of oxyresveratrol	
		AUC /AUC IS	AUC /AUC IS			Based on weight of extract	Based on weight of heartwood
Acetone 8 hrs RT	1	3.754084	3.775797	3.764941	0.150342	79.55	3.15
	2	3.057502	3.070901	3.064201	0.11921	67.73	2.89
	3	3.562412	3.584984	3.573698	0.141845	79.69	2.90
EtOAc 8 hrs RT	1	3.890143	3.891401	3.890772	0.155932	93.93	2.20
	2	4.313193	4.298726	4.30596	0.174377	97.42	2.22
	3	4.327724	4.309719	4.318722	0.174944	98.28	2.22
EtOH 48 hrs RT	1	3.152894	3.153109	3.153001	0.123155	61.27	3.03
	2	3.579662	3.572826	3.576244	0.141959	70.98	3.82
	3	3.508278	3.485524	3.496901	0.138434	71.73	3.91
Acetone 48 hrs RT	1	3.343861	3.271549	3.307705	0.130028	76.04	3.58
	2	3.382705	3.364171	3.373438	0.132949	79.14	3.99
	3	2.956708	2.923693	2.9402	0.113701	60.16	3.53
EtOAc 48 hrs RT	1	4.230739	4.200369	4.215554	0.170361	96.25	2.58
	2	3.559621	3.589368	3.574494	0.141881	87.58	2.48
	3	4.540865	4.578597	4.559731	0.185652	103.72	3.07
Puag-Haad	1	1.193864	1.200931	1.197397	0.09068	78.18*	
	2	1.248804	1.239182	1.243993	0.09586	94.91*	
	3	1.223305742	1.204598377	1.21395206	0.037009732	91.61*	

*- adjusted to 25 ml

7 Structure modification of oxyresveratrol

6.1 Preparation of oxyresveratrol tetra-ethylcarbonate (AS-1)

Oxyresveratrol (51.1 mg, 0.209 mmol) was dissolved in methylenechloride (2 ml) in a round bottom flask with a magnetic bar. The flask was placed in an ice-bath. Then triethylamine (0.18 ml, 1.3 mmol), DMAP (103.7 mg, 0.85 mmol) and ethylchloroformate (0.12 ml, 1.3 mmol) were added, respectively. The reaction was monitored by TLC (Silica gel, CH_2Cl_2) and completed within an hour. Extraction with methylenechloride and purification by column chromatography (Silica gel, 7734, CH_2Cl_2) resulted in 64.1 mg (0.120 mmol) of AS-1 (57.55%).

AS-1: UV (MeOH): λ_{max} nm (ε) 226 (22312), 297 (34381); Figure 29

IR (UATR): ν_{max} cm^{-1}1756 , 1215; Figure 30

ESIMS: *m/z* 532 $[M]^+$ Figure 31; TOF MS: *m/z* 533.1656 (found) 533.1660 (Calculated for $C_{26}H_{29}O_{12}$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 32

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 33

6.2 Preparation of oxyresveratrol tetra-diethylcarbamate (AS-2)

Oxyresveratrol (50.9 mg, 0.209 mmol) was dissolved in methylenechloride (2 ml) and placed in an ice-bath. Then triethylamine (0.17 ml, 1.3 mmol), DMAP (101.94 mg, 0.83 mmol) and diethylchlorocarbamate (0.16 ml, 0.13 mmol) were added respectively. The mixture was stirred at room temperature overnight. The reaction was monitored by TLC (silica gel, CH_2Cl_2) and completed within 24 hours. Extraction with CH_2Cl_2 and purification by preparative TLC (Silica gel, 7734 CH_2Cl_2) resulted in 74.1 mg (0.116 mmol) of AS-2 (55.51%).

AS-2 : UV (MeOH): λ_{max} nm (ε) 299 (24428); Figure 34

IR (UATR): ν_{max} cm^{-1}; 1710, 1145; Figure 35

ESIMS: *m/z* 640 $[M]^+$; Figure 36; TOF MS: *m/z* 641.3537 (found) 641.3551 (Calculated for $C_{34}H_{49}N_4O_8$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 37 and

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 38

6.3 Preparation of oxyresveratrol tetra-acetate (AS-3)

Oxyresveratrol (51.0 mg, 0.209 mmol) was dissolved in methylenechloride (2 ml) and placed in an ice-bath. Then triethylamine (0.17 ml,1.3 mmol), DMAP (103.7 mg, 0.85 mmol) and chloroacetate (0.09 ml, 0.13 mmol)were added respectively. The mixture was stirred at room temperature for 2 hours. The reaction was monitored by TLC (silica gel, CH_2Cl_2). Extraction with CH_2Cl_2 and purification by preparative TLC (Silica gel, 7734 CH_2Cl_2) resulted in 74.1 mg (0.18 mmol) of AS-3 (86.07%).

AS-3 : UV (MeOH): λ_{max} nm (ε) 226 (18611), 297 (25678); Figure 39

IR(UATR): ν_{max} cm^{-1}; 1756, 1192, Figure 40

ESIMS: *m/z* 412 $[M]^+$; Figure 41; TOF MS: *m/z* 413.1229 (found)

413.1237 (calculated for $C_{22}H_{21}O_8$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16Figure 42 and

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 43

6.4 Preparation of oxyresveratrol tetra-benzoate (AS-4)

Oxyresveratrol (36.3 mg, 0.15 mmol) was dissolved in methylenechloride (2 ml) and placed in an ice-bath. Then triethylamine (0.12 ml, 0.9 mmol), DMAP (73.4 mg, 0.6 mmol) and benzoylchloride (0.1 ml, 0.9 mmol) were added respectively. The mixture was stirred at room temperature. The reaction was monitored by TLC (silica gel, CH_2Cl_2) and completed within 2 hours. Extraction with CH_2Cl_2 and purification by preparative TLC (Silica gel, 7734 CH_2Cl_2) resulted in 60.7 mg (0.09 mmol)of AS-4 (61.83%).

AS-4: : UV (MeOH): λ_{max} nm (ε) 242 (55460), 285 (38311); Figure 44

IR (UATR): ν_{max} cm^{-1} 1728, 1259 Figure 45

ESIMS; *m/z* 660 $[M]^+$ Figure 46; TOF MS: *m/z* 661.1841 (found)

661.1863 (calculated for $C_{42}H_{29}O_8$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 47

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 48

6.5 Preparation of tetra-*O*-phenylmethyl oxyresveratrol (AS-5)

Sodium hydride (36.88 mg, 0.922 mmol) was placed in a round-bottom flask with magnetic bar. Then dimethylformamide was added into flask rapidly, followed by oxyresveratrol (50 mg, 0.205 mmol). The mixture was stirred in an ice-bath for 30 minutes. After that benzylbromide (0.15 ml, 1.26 mmol) was added and stirred overnight. The reaction was monitored by TLC (Silica gel, CH_2Cl_2) and completed within 24 hours. Extraction with CH_2Cl_2 and purification by column chromatography (Silica gel 7734, CH_2Cl_2) resulted in 46.1 mg (0.076 mmol)of AS-5 (37.24%, mmol).

AS-1: UV (MeOH): λ_{max} nm (ε) 243 (22250), 329 (32909); Figure 49

IR (UATR): ν_{max} cm^{-1} 3032, 2865, 1595; Figure 50

TOF MS: *m/z* 605.2688 (found) 605.2692 (calculated for $C_{42}H_{37}O_4$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 51

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 52

6.6 Preparation of tetra-*O*-methyl oxyresveratrol (AS-6)

Oxyresveratrol (50.0 mg, 0.205 mmol) was dissolved with acetone (2 ml). To this solution potassium carbonate (169.9 mg, 1.23 mmol) and dimethylsulfate (0.81 ml, 8.6 mmol) was added. The reaction was monitored by TLC (silica gel, CH_2Cl_2) and completed within 24 hours. Extraction with CH_2Cl_2 and purification by preparative TLC (Silica gel, 7734 CH_2Cl_2) resulted in 70.6 (0.235 mmol) mg of AS-6 (114.84 %).

AS-6 was also prepared by methylation with methyl iodide. Oxyresveratrol (51.1 mg, 0.21 mmol) was dissolved in acetone (10 ml). Then potassium carbonate (181.9 mg, 1.31 mmol) and methyl iodide (0.076 ml, 1.23 mmol) were added respectively. The mixture was refluxed at 65°C-75°C overnight. The reaction was monitored by TLC (Silica gel, CH_2Cl_2). After the reaction was completed, the mixture was extracted with CH_2Cl_2 and purified by column chromatography and preparative TLC, to give 49.16 mg (0.1639 mmol) of AS-6 (78.04 %).

AS-1: UV (MeOH): λ_{max} nm (ε) 217 (12508), 300 (9966), 325 (13983);
Figure 53

IR (UATR): ν_{max} cm^{-1} 2937, 2835, 1587; Figure 54

TOF MS: *m/z* 301 $[M+H]^+$ Figure 55; TOF MS: *m/z* 323.1263 (found)
323.1262 (calculated for $C_{18}H_{21}O_4$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 56

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 57

6.7 Preparation of dihydrooxyresveratrol (AS-7)

Oxyresveratrol (100 mg, 0.410 mmol) was dissolved in EtOH (10 ml) with Palladium on charcoal (3.0 mg) in a Parr apparatus filled with hydrogen gas at pressure 47 bar. The mixture was shaken at room temperature for an hour. The reaction was monitored by TLC (silica gel, 10% MeOH in CH_2Cl_2). After the reaction was completed, the mixture was filtered through kieselguhr and dried under reduced pressure to give 98.84 mg (0.402 mmol) of AS-7 (98.05%).

AS-7: UV (MeOH): λ_{max} nm (ε) 280 (4141); Figure 58

IR (UATR): ν_{max} cm^{-1} 3204, 1604, Figure 59

ESIMS: *m/z* 246 $[M]^+$ Figure 60; TOF MS: *m/z* 247.0962 (found) 247.0971 (calculated for $C_{14}H_{15}O_4$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 61

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 62

6.8 Preparation of 2,3′-di-*O*-methyl oxyresveratrol (AS-8), 4,3′-di-*O*-methyl oxyresveratrol (AS-9) and 2-*O*-methyl oxyresveratrol (AS-10)

Oxyresveratrol (501.0 mg, 2.05 mmol) was dissolved in acetone (10 ml) Then potassium carbonate (853.6 mg, 6.17 mmol) and methyliodide (0.38 ml, 6.15 mmol) were added respectively. The mixture was refluxed at 65°C-75°C for 2 hours. The reaction was monitored by TLC (Silica gel, CH_2Cl_2). After the reaction was completed, the mixture was extracted with CH_2Cl_2 and purified by column chromatography and preparative TLC to give 43.7 mg (0.1457 mmol) of 2,3′-di-*O*-methyl oxyresveratrol (7.11 %) [AS-8], 15.9 mg (0.0585 mmol) of 4,3′-di-*O*-methyl oxyresveratrol (2.85 %) [AS-9], 19.8 mg (0.1465 mmol) of 2-*O*-methyl oxyresveratrol (7.14%) [AS-10].

AS-8: UV (MeOH): λ_{max} nm (ε) 300 (7382), 325 (9792); Figure 63

IR (UATR): ν_{max} cm^{-1} 3361, 1589; Figure 64

ESIMS: *m/z* 272 $[M+H]^+$ Figure 65; TOF MS: *m/z* 273.1121 (found) 273.1128 (calculated for $C_{16}H_{17}O_4$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 66

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 67

AS-9: UV (MeOH): λ_{max} nm (ε) 300 (7315), 327 (9829) ; Figure 69

IR (UATR): ν_{max} cm^{-1} 3374, 2918, 1590; Figure 70

ESIMS: *m/z* 272 $[M+H]^+$ Figure 71; TOF MS: *m/z* 295.0946 (found) 295.0948 (calculated for $C_{16}H_{16}O_4Na$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 72

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 73

AS-10: UV (MeOH): λ_{max} nm (ε) 217 (10581), 300 (7373), 326 (10686); Figure 75

IR (UATR): ν_{max} cm^{-1} 1756, 3331, 1600; Figure 76

ESIMS: *m/z*; 258 $[M+H]^+$ Figure 77; TOF MS: *m/z* 281.0789 (found)

281.0792 (calculated for $C_{15}H_{14}O_4Na$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 78

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 79

6.9 Preparation of 2′-chloro-oxyresveratrol (AS-11)

Oxyresveratrol (50 mg, 0.205 mmol) was dissolved in dry acetic acid (2 ml). Then N-chlorosuccinimide 27.4 mg (0.205 mmol)was added. The reaction was stirred over night at room temperature under Argon. The reaction was monitored by TLC (Silica gel, 15% MeOH in CH_2Cl_2) and was completed within 24 hours. Extraction with MeOH and purification by column chromatography and MPLC (Silica gel 7734, CH_2Cl_2) resulted in 29.1 mg (0.105 mmol) of AS-11 (51.08%).

AS-11: UV (MeOH): λ_{max} nm (ε) 218 (30568), 332 (29331); Figure 81

IR (UATR): ν_{max} cm^{-1} 3375, 3286, 1598; Figure 82

ESIMS: *m/z*; 278 $[M]^+$ Figure 83; TOF MS: *m/z* 279.0426 (found) 279.0425

(calculated for $C_{14}H_{13}ClO_4$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 84

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 85

6.10 Preparation of *cis*-tetra-*O*-methyl oxyresveratrol (AS-12)

AS-6 (68.2 mg, 0.2273 mmol) was dissolved in benzene (0.6 ml) in a small test tube and was exposed to sunlight for a total of 40 hours (8 hours/day for 5 days). The solution which was clear on the first day gradually turned yellow. The progress of reaction was moitored by TLC. After the reaction reached an equilibrium, the mixture was chromatographed on aluminium oxide TLC plates with 10% diethyl ether in petroleum ether as the developing solvent (triple development) to give 22.1 mg (0.0737 mmol) of AS-12 (32.40%). The product *cis*-tetra-*O*-methyl oxyresveratrol (AS-12) showed

a quenching spot under UV light at 254 nm but did not fluoresce at 428 nm as opposed to the starting material (AS-6), the *trans*-isomer, which gave a fluorescent spot at 428 nm).

AS-12: UV (MeOH): λ_{max} nm (ε) 280 (3832); Figure 87

IR (UATR): ν_{max} cm^{-1} 1588; Figure 88

ESIMS: *m/z* 323 $[M+Na]^+$ Figure 89 TOF MS: *m/z* 323.1268 (found) 323.1262 (calculated for $C_{18}H_{20}O_4Na$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 90

^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 91

6.11 Preparation of tetra-*O*-methyl dihydrooxyresveratrol (AS-13)

AS-6 (60 mg, 0.2 mmol) was dissolved in EtOAc (10 ml) with Palladium on charcoal (2.6 mg) in a Parr apparatus filled with hydrogen gas at pressure 47 bar. The mixture was shaken at room temperature for an hour. The reaction was monitored by TLC (silica gel, 20% hexane in CH_2Cl_2). After the reaction was completed, the mixture was filtered through kieselguhr and the solvent was evaporated under reduced pressure to give 59.1 mg (0.196 mmol) of AS-13 (97.86%).

AS-13: UV (MeOH): λ_{max} nm (ε) 278 (35789); Figure 94

IR: ν_{max} cm^{-1} 1588; Figure 95

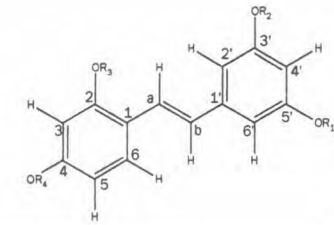
ESIMS: *m/z*; 302 $[M]^+$ Figure 96; TOF MS: *m/z* 303.1592 (found) 303.1597 (calculated for $C_{18}H_{23}O_4$)

^{1}H-NMR (300 MHz, $CDCl_3$): Table 16; Figure 97

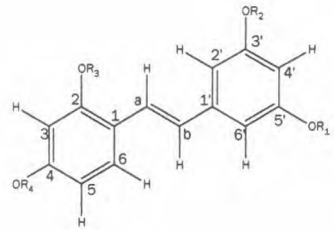
^{13}C-NMR (75 MHz, $CDCl_3$): Table 17; Figure 98

Table 16: ^{1}H-NMR data

A: ^{1}H-NMR data for stilbene skeleton of synthesized compounds



Position	δ (ppm) (multiplicity, *J* in Hz) ($CDCl_3$)												
H	AS-1	AS-2	AS-3	AS-4	AS-5	AS-6	AS-7	AS-8	AS-9	AS-10*	AS-11**	AS-12	AS-13
3	7.12 (br s)	7.00 (br s)	6.93 (br s)	Complex and	Complex 6.58 (m)	6.50 (br s)	6.25 (d, 1.7)	6.37 (br s)	6.363 (d, 2.4)	6.45 (br s)	6.32 (d, 2.5)	6.42 (br s)	6.25 (d, 2.4)
5	7.09 (br d, 8.4)	6.97 (br d, 8.4)	7.00 (br d, 8.1)	unresolved signals		6.54 (br d, 8.6)	6.09 (dd, 8.1, 1.7)	6.39 (br d, 8.4)	6.50 (dd, 8.7, 2.4)	6.42 (br d, 8.5)	6.41 (dd, 8.5, 2.5)	6.25 (dd, 8.4, 2.4)	6.39 (dd, 8.1, 2.4)
6	7.60 (d, 8.4)	7.55 (d, 8.4)	7.59 (d, 8.1)	from 7.10-8.30	unresolved signals from 7.24-7.54	7.53 (d, 8.6)	6.78 (d, 8.1)	7.36 (d, 8.4)	7.39 (d, 8.7)	7.41 (d, 8.5)	7.45 (d, 8.5)	7.14 (d, 8.4)	6.98 (d, 8.1)
2′	7.19 (br s)	7.04 (br s)	7.06 (br s)		6.71 (br s)	6.71 (br s)	6.05 (br s)	6.55 (br s)	6.61 (br s)	6.48 (br s)	-	6.43 (br s)	6.35 (br s)
4′	7.02 (br s)	6.88 (t, 1.8)	6.88 (br s)		6.49 (t, 1.8)	6.40 (br s)	5.99 (br s)	6.25 (br s)	6.30 (t, 1.8)	6.03 (br s)	6.37 (d, 3)	6.27 (t, 2.1)	6.29 (t, 2.3)
6′	7.19 (br s)	7.04 (br s)	7.06 (br s)		6.71 (br s)	6.71 (br s)	6.05 (br s)	6.55 (br s)	6.58 (br s)	6.48 (br s)	6.77 (d, 3)	6.43 (br s)	6.35 (br s)
α	7.142 (d, 16.2)	7.12 (d, 16.2)	7.01 (overlapping with β)		unresolved signals	7.40 (d, 16.4)	2.55 (m)	7.27 (d, 16.2)	7.21 (d, 16.2)	7.25 (d, 16.5)	7.24 (d, 16.2)	6.61 (d, 12.3)	2.79 (m)
β	6.989 (d, 16.2)	6.93 (d, 16.2)	6.97 (overlapping with α)		6.95 (d, 16.5)	6.97 (d, 16.4)	2.55 (m)	6.81 (d, 16.2)	6.87 (d, 16.2)	6.83 (d, 16.5)	7.20 (d, 16.2)	6.46 (d, 12.3)	2.79 (m)



B: ^{1}H-NMR data for substituents on stilbene skeleton of synthesized compounds

Position	δ (ppm) (multiplicity, *J* in Hz) ($CDCl_3$)											
	AS-1	AS-2	AS-3	AS-4	AS-5	AS-6	AS-7	AS-8	AS-9	AS-10*	AS-12	AS-13
R_1	CH_3 1.35-1.40 (m) **CH_2 4.28-4.35 (m)**	CH_3 1.18 (br s) CH_2 3.37 (br s)	2.89 (s)	Phenolics complex and unresolved signal from 7.1-8.3	Phenolics complex and unresolved signal from 7.0-7.3 CH_2 5.09 (br s), 5.03 (br s)	3.62 (s)	9.01 (s)				3.63 (s)	3.76 (s)
R_2								3.79 (s)	3.79 (s)			
R_3			2.28 (s)			3.77 (s)	8.94 (s)	3.76 (s)		3.79	3.77 (s)	3.78 (s)
R_4			2.34 (s)			3.79 (s)	9.05 (s)		9.78 (s)		3.80 (s)	3.80 (s)

Table 17: ^{13}C-NMR data

A: ^{13}C -NMR data for stilbene skeleton of synthesized compounds

Position	δ (ppm) ($CDCl_3$)												
	AS-1	AS-2	AS-3	AS-4	AS-5	AS-6	AS-7	AS-8	AS-9	AS-10*	AS-11**	AS-12	AS-13
1	127.5	126.6	127.0	127.9	120.5	119.3	119.3	118.2	117.3	118.4	116.6	119.2	122.5
2	149.1	149.0	148.3	149.3	157.6	157.9	156.5	157.9	154.0	158.8	152.9	158.7	158.1
3	115.9	116.4	116.3	117.2	101.7	99.4	103.4	100.3	101.8	102.3	102.5	99.9	98.5
4	151.3	151.1	150.4	151.3	160.0	160.4	157.1	158.0	160.2	159.1	157.6	160.8	159.1
5	119.6	118.9	119.4	120.3	107.2	105.0	106.8	107.5	106.9	108.3	104.1	104.7	103.7
6	129.9	128.7	129.4	134.3	128.5	127.3	130.8	127.2	128.1	127.7	127.5	131.2	129.9
1′	139.9	139.1	127.3	140.0	140.8	140.3	145.2	140.4	140.1	141.2	137.5	119.1	144.8
2′	117.2	116.3	116.9	117.8	106.1	104.4	106.8	103.7	104.7	105.7	107.7	107.0	160.5
3′	152.1	151.9	151.2	152.0	160.5	160.7	159.0	160.6	160.9	159.2	155.6	160.8	160.8
4′	114.3	114.8	114.7	115.6	101.5	98.5	101.0	99.0	100.6	99.7	101.9	98.8	97.7
5′	152.1	151.9	151.2	152.0	160.5	160.7	159.0	160.6	156.6	159.2	155.5	160.8	160.4
6′	117.2	116.3	116.9	117.8	106.1	104.4	106.8	105.8	105.7	105.7	110.7	107.0	106.5
α	127.7	133.2	123.4	124.0	124.5	123.8	32.2	123.6	123.4	123.7	121.6	126.2	31.7
β	127.7	126.3	127.1	130.1	127.6	126.9	37.2	126.2	127.5	126.6	125.7	129.3	36.8

B: ^{13}C-NMR data for substituents on stilbene skeleton of synthesized compounds

Position	δ (ppm) ($CDCl_3$)										
	AS-1	AS-2	AS-3	AS-4	AS-5	AS-6	AS-8	AS-9	AS-10*	AS-12	AS-13
R_1	CH_3 14.9, CH_2 65.7, 65.9 OCOO 153.5, 153.3, 153.4	CH_3 13.5, 14.4 CH_2 42.0, 42.3 OCON 153.4, 153.6	21.1, 21.2 168.6	Phenolics complex and unresolved signal from 127-134, OCO 165.0	Phenolics complex and unresolved signal from 127-129, CH_2 70.6	55.4	55.5	55.2		55.6	55.3,55.4
R_2											
R_3					CH_2 70.7	55.5	55.3		55.6	55.7	
R_4					CH_2 70.8	55.6		55.2		55.9	

* run in deuterated acetone

** run in $CDCl_3$+CD_3OD

7 Tyrosinase activity

7.1. Materials and instruments

L-DOPA	Sigma Chemical Co.
Tyrosinase	Sigma Chemical Co.
Kojic acid	Sigma Chemical Co.
Sodium dihydrogen phosphate	May & Baker Ltd.
Disodium hydrogen phosphate	May & Baker Ltd.
Water	Highly purified water 18.2 mΩ
96-well plate	greiner cell star®
Microplate reader	Perkin Elmer - Victor^3
Statistical Analysis –software	Microsoft® Office Excel 2003
Graph –SigmaPlot 2000 Version 6.10	

7.2. Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined by the dopachrome method using L-DOPA as the substrate (Iida *et al.*, 1995). Dopachrome is one of the intermediates in the melanin biosynthesis. The pink color of dopachrome can be detected by visible light. In this experiment a microplate reader (Perkin Elmer - Victor^3) with a 490 nm interference filter was used for detection. The potential tyrosinase inhibitor could show minimal dopachrome absorption. This method was modified from the methods of Masamoto (Masamoto *et al.*, 1980), Iida (Iida *et al.*, 1995) and Morita (Moritea *et al.*, 1994).

7.3. Preparation of the reaction mixture

7.3.1. Preparation of 20 mM phosphate buffer (pH 6.8)

Solution A: NaH_2PO_4 $2H_2O$ (312 mg) was dissolved in 100 ml of H_2O. Solution B: Na_2HPO_4 (284 mg) was dissolved in 100 ml of H_2O. Then, portions of solutions A and B were slowly mixed until pH 6.8 was reached.

7.3.2. Preparation of 0.85 mM L-DOPA

L-DOPA (0.8 mg) was dissolved in 5 ml of 20 mM phosphate buffer (pH6 .8).

7.3.3. Preparation of tyrosinase solution

Accurately weighed tyrosinase enzyme (1.5 mg) was dissolved in 10 ml of 20 mM phosphate buffer (pH 6.8).

7.3.4. Preparation of the test sample

Accurately weighed test sample was dissolved in ethanol in a volumetric flask. The unit of concentration, initially obtained as mg/ml, was expressed as µM in the final calculation. For example, the concentration of oxyresveratrol (MW 244) at 1 mg/3 ml was converted to the unit of molar (M) by dividing this value with its molecular weight ([1 mg/3 ml] / 244 = 0.001366 M = 1366 µM). For the IC_{50} determination, serial dilution was performed with ethanol until a set of sample solutions with suitable range of concentrations was obtained. The total volume of the reaction mixture was 200 µl.

7.4. Measurement of activity

7.4.1. Substrate-inhibitor pre-incubation method

The reaction mixture (200 µl) was measured in four wells (A, B, C and D). In each well, the substance was added in order of mixing, as follows;

A (Control)	40 µl of 0.85 mM L-DOPA
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of ethanol

B (blank of A)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of ethanol
C (Test sample)	40 µl of 0.85 mM L-DOPA
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test sample in ethanol
D (blank of C)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test sample in ethanol

The solution in each well was mixed and pre-incubated at 25°C for 10 minutes. Then 40 µl of mushroom tyrosinase solution (240 unit/ml) was added and the mixture was incubated at 25°C for 20 minutes. The absorbance of each well was measured at 490 nm with the microplate reader both before and after incubation.

$$\% \text{ inhibition} = [(B-A)-(D-C))/(B-A)] \times 100$$

A = Positive control

B = Blank of positive control

C = Test sample

D = Blank of C

$SE = SD/\sqrt{N}$

SE = Standard error

SD = Standard deviation

N = Number of measurement

7.4.2. Enzyme-inhibitor pre-incubation method

The reaction mixture (200 µl) was measured in four wells (A, B, C and D). In each well, the substance was added in order of mixing, as follows;

A (Control)	40 µl of mushroom tyrosinase solution (240 unit/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of ethanol
B (blank of A)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of ethanol
C (Test sample)	40 µl of mushroom tyrosinase solution (240 unit/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test sample in ethanol
D (blank of C)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test sample in ethanol

The solution in each well was mixed and pre-incubated at 25°C for 10 minutes. Then, 40 µl of 0.85 µl L-DOPA was added and incubated at 25°C for 20 minutes. The absorbance of each well was measured at 490 nm with the microplate reader both before and after incubation.

7.5. Preliminary evaluation of tyrosinase activity

The substrate-inhibitor pre-incubation method was employed. The final concentration of the test sample in each well was 100 µM. Activity was measured and expressed as percent inhibition. Samples showing more than 80% inhibition at this concentration will be further analyzed for the IC_{50} value.

7.6. Determination of IC_{50}

The IC_{50} value is the concentration of the test sample that can cause 50% inhibition of DOPA oxidase activity of the enzyme. An IC_{50} value was obtained by plotting % inhibitions versus concentrations of the inhibitor. This was performed on a set of sample solutions with various concentrations prepared by the serial dilution method.

7.7. Enzyme kinetics

Enzymes kinetics was performed using the substrate-inhibitor pre-incubation method. Measurement of the initial rate of dopachrome formation of the reaction mixture was expressed as the increase of absorbance at wavelength 490 nm per min (ΔA_{492}/min). The Michaelis-Menten constant (Km) and maximal velocity (Vmax) of tyrosinase were determined by Lineweaver-Burk plot with various concentrations of L-DOPA as the substrate.

The experiment was carried out in triplicate using a series of L-DOPA solutions against two concentrations of the test sample in comparison with the control. First, L-DOPA was accurately weighed (9.86 mg) and then dissolved in 10 ml of 20 mM phosphate buffer to give 5 mM L-DOPA solution. This solution (5 ml) was then transferred to a volumetric flask and the final volume of the solution was adjusted to 10 ml to give 2.5 mM L-DOPA solution. Further serial dilutions were carried out in a similar manner to give 1.25 mM and 0.625 mM solutions of L-DOPA. In additon, 1 ml of 5 mM L-DOPA solution was diluted with the buffer to give 6 ml of 0.83 mM L-DOPA solution. In the test, 40 µl of the L-DOPA solutions of 5 mM, 2.5 mM, 1.25 mM, 0.83 mM and 0.625 mM was added to the reaction mixture of each well of the microplate to give the final concentrations of L-DOPA as 1, 1/2, 1/4, 1/6 and 1/8 mM, respectively.

The two concentrations of the test sample selected for the kinetic study were one- and two-folds of the IC_{50} value. The absorbance was taken every second for one minute Then the inverse of V versus the inverse of the substrate concentration were plotted in Lineweaver-Burk graph model.

From the Lineweaver-Burk plot, Km Vmax and Ki were obtained from the below equations

$$\text{X-intercept} = -1/K_m$$

K_m = Michaelis-Menten constant

$$\text{Y-intercept} = 1/V_{max}$$

Vmax = the velocity at maximal concentrations of substrate

$K_i = [I]/((V_{max}/V_{max'}) - 1)$

Where

K_i = the dissociation constant for inhibitor binding

[I] = concentration of inhibitor

V_{max} = The maximum enzyme velocity

$V_{max'}$ = The enzyme velocity with the presence of inhibitor

8 Cytotoxicity assays

Cytotoxicity was performed on three cancer cell lines (1) KB (Human epidermoid carcinoma of cavity, ATCC CCL-17) (2) BC (Breast cancer) and (3) NCI-H187 (Human small cell lung carcinoma, ATCC CRL-5804). The tests were performed by the Bioassay Laboratory, National Center of Genetic Engineering and Biotechnology (BIOTEC) of the National Science and Technology Development Agency (NSTDA). The procedures were published previously (Plumb, Milroy and Kaye, 1989; Skehan, Ritsa and Dominic, 1990). KB and BC were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content according to Skehan, Ritsa and Dominic (1990). Elliptine and doxorubicin were used as positive controls. DMSO was used as negative control. Briefly, cells at a logarithimic growth phase were harvested and diluted to 10^5 cells/ml with fresh medium and gently mixed. Test compounds were diluted in distilled water and put into microtiter plates in total volume of 200 ul. Plates were incubated at $37^{\circ}C$, 5% CO_2 for 72 h. After incubation period, cells were fixed by 50% trichloroacetic acid. The plates were incubated at $4^{\circ}C$ for 30 min, washed with tap water and air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B (SRB) dissolved in 1% acetic acid for 30 min. After the staining period, SRB was removed with 1% acetic acid. Plates were air-dried before bound dye was solubilized with 10 mM Tris base for 5 min on a shaker. OD was read in a microtiter plate reader at wavelength of 510 nm.

Cytotoxicity against NCI-H187 was determined by MTT assay previously described in detail by Plumb *et al.* Briefly, cells were diluted to 10^5 cells/ml. Test compounds were diluted in distilled water and added to microtiter plates in total volume of 200 ul. Plates were incubated at 37°C under 5% CO_2 for 5 days. 50 ul of 2 mg/ml MTT solution (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) was added to each well of the plate. Plates were wrapped with aluminium foil and incubated for 4 h. After incubation, the micro plates were spinned down at 200 x g for 5 min. MTT was then removed from the wells and the formazan crystals were dissolved in 200 µl of 100% DMSO and 25 µl of Sorensen's glycine buffer. OD was read in microtiter plate reader at wavelength of 510 nm.