Metabolomic identification of anticancer metabolites of Australian propolis and proteomic elucidation of its synergistic mechanisms with Doxorubicin in the MCF7 cells

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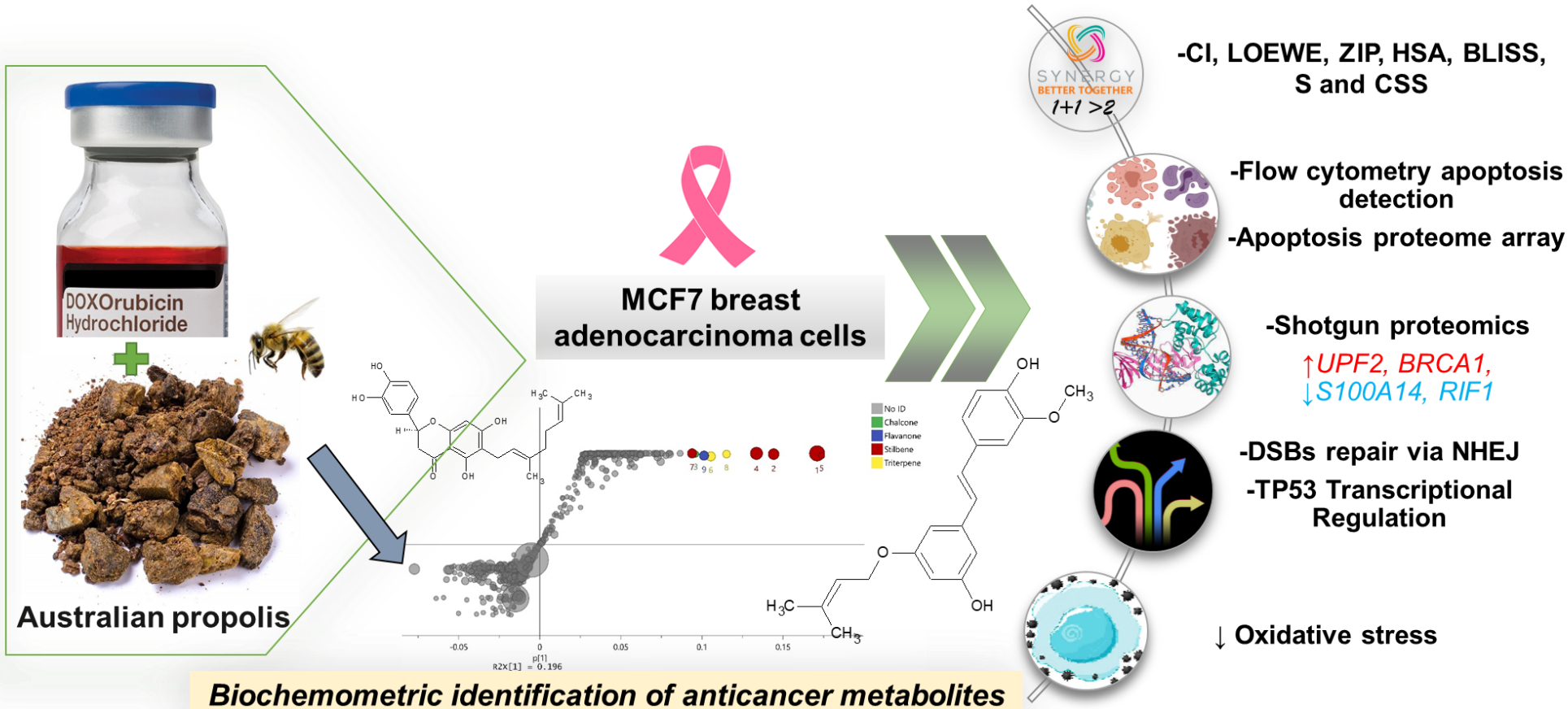

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Table S1 Metabolomic-driven identified active metabolites of propolis samples against MCF-7 cells using UPLC-qTOF-MS and chemometrics

CPD ID	m/z	Rt (min)	CCS (A^2)	Accepted Identification	Adducts*	Formula	Class	MS^n	Mass Error (ppm)
1	341.1358	16.71	181.5785	Prenyl-tertrahydroxy-methoxy-stilbene (5,2',3',4'-tetrahydroxy-3-methoxy-2-prenyl-(E)-stilbene)	M-H	C20H22O5	Stilbene	**341.1419(100%),** 323.1281, 244.0374, 242.0561, **188.0480**, 144.0580	-10.58
2	341.1374	17.29	181.5784	Prenyl-tetrahydroxy-methoxy-stilbene (5,6,3',4'-tetrathydroxy-3-methoxy-2-prenyl-(E)-stilbene)	M-H	C20H22O5	Stilbene	341.1406, 323.1273, 244.0374, 242.0571, **188.0479 (100%),** 144.0579	-5.99
3	323.1264	18.17	183.7832	Prenyl-trihydroxy-chalcone	M-H	C20H20O4	Chalcone	323.1281(100%), 255.0615 254.0567, 226.0616	-7.63
4	325.1337	18.22	181.9172	Trihydroxy-methoxy-prenyl-(E)-stilbene	M-H	C20H22O4	Stilbene	325.1468, 272.0685, 241.0497, **188.0491 (100%),** 144.0583	-14.74
5	325.1417	18.79	180.0948	Dihydroxy-methoxy-prenyloxy-(E)-stilbene	**M-H**, M-H2O-H	C20H22O4	Stilbene	325.1477, 241.0503, **188.0499 (100%),** 144.0587	-8.61
6	489.3525	19.00	229.2606	Undefined triterpene	M-H2O-H, **M-H**	C30H50O5	Triterpene	M-H (489.3601 (100%), 235.1326, 207.1019, M-H2O-H (471.3428,235.1327, 207.1020, 144.9221)	-14.72
7	379.1881	19.14	213.2551	Bisprenyl-tetrahydroxystilbene [(E)-2,6-bis(3-methyl-2-buten-1-yl)-3,3',5,5'-tetrahydroxystilbene]	M-H2O-H, **M-H**, M+FA-H	C24H28O4	Stilbene	379.1935 (100%), 309.1111, 323.1289, 267.0652, 255.0663, 254.0558, 257.1533	-8.92
8	489.3535	19.68	229.2606	Undefined triterpene	M-H	C30H50O5	Triterpene	489.3598(100%), 235.1329, 207.1017	-10.23
9	423.1806	20.94	210.7108	Geranyl-tetrahydroxyflavanone (propolin C/D/F)	M-H2O-H, **M-H**, M+Na-2H, M+K-2H	C25H28O6	Flavanone	423.1822 (M-H), 287.1306 (100%), 135.0472 (95%)	-8.66

CCS; collision cross-section, *; Bold adducts are the most abundant ones with the reported precursor ion m/z and CCS.

Table S2 Pearson's correlation of different DrugComb metrics and CI values at different in inhibitory concentration

	CI at				
	ED50	ED75	ED90	ED95	ED97
CSS	0.96	0.89	0.83	0.78	0.75
S	0.95	0.88	0.83	0.78	0.75
ZIP	0.83	0.78	0.74	0.71	0.68
BLISS	0.83	0.78	0.74	0.71	0.68
LOEWE	0.81	0.75	0.70	0.67	0.64
HSA	0.87	0.81	0.76	0.72	0.69

Table S3 Percentage of necrotic, apoptotic, and living cells retrieved from Annexing V-7AAD flowcytometry apoptosis analysis among propolis, doxorubicin and their mixture

	% Necrotic cells	% late apoptotic cells	%living cells	% Early apoptotic cells
Negative Control	0.56±0.32[a]	8.7±2.39[a]	87.63±2.52[a]	3.12±0.35 [a]
Propolis	3.41±0.842[a]	43.53±12.89[b]	10.04±8.22[b]	43.02±5.46[b]
Doxorubicin	83.85±3.15[b]	13.46±3.38 [a]	2.16±0.44 [b]	0.53±0.09[a]
Combination	4.25±4.04[a]	87.59±7.44[c]	6.39±3.78 [b]	1.77±0.91[a]

Superscript letters indicate statistical significance derived from two-way ANOVA and Tukey's multiple comparisons where different letters within the same column are statistically significant with $P<0.0001$, n=4.

Table S4 Human apoptosis array coordinates and corresponding proteins

Coordinates	Protein	Coordinates	Protein
A1, A2	**Reference Spots1**	*C17, C18*	HSP60
A23, A24	**Reference Spots2**	*C19, C20*	HSP70
B1, B2	Bad	*C21, C22*	HTRA2/Omi
B3, B4	Bax	*C23, C24*	Livin
B5, B6	Bcl-2	*D1, D2*	PON2
B7, B8	Bcl-x	*D3, D4*	p21/CIP1/CDKN1A
B9, B10	Pro-Caspase-3	*D5, D6*	p27/Kip1
B11, B12	Cleaved Caspase-3	*D7, D8*	Phospho-p53 (S15)
B13, B14	Catalase	*D9, D10*	Phospho-p53 (S46)
B15, B16	cIAP-1	*D11, D12*	Phospho-p53 (S392)
B17, B18	cIAP-2	*D13, D14*	Phospho-Rad17 (S635)
B19, B20	Claspin	*D15, D16*	SMAC/Diablo
B21, B22	Clusterin	*D17, D18*	Survivin
B23, B24	Cytochrome c	*D19, D20*	TNF RI/TNFRSF1A
C1, C2	TRAIL R1/DR4	*D21, D22*	XIAP
C3, C4	TRAIL R2/DR5	***D23, D24***	**PBS (Negative Control)**
C5, C6	FADD		
C7, C8	Fas/TNFRSF6/CD95		
C9, C10	HIF-1α		
C11, C12	HO-1/HMOX1/HSP32		
C13, C14	HO-2/HMOX2	***E1, E2***	**Reference Spots3**
C15, C16	HSP27		

Table S5 Significant apoptotic proteins in MCF-7 cell lysate upon treatment by doxorubicin, propolis or its synergistic combination

Dysregulated proteins* & pairwise comparisons	p value	-Log10(p)	FDR	Fold Change	log2(FC)
Doxorubicin vs control					
Bcl-x	0.000385	3.42	0.000923	0.32	-1.63
Claspin	0.000345	3.46	0.000923	0.43	-1.21
Bad	0.000117	3.93	0.000453	0.52	-0.95
Pro-Caspase-3	1.89E-07	6.72	3.31E-06	0.58	-0.80
Survivin	0.000105	3.98	0.000453	0.59	-0.75
Cleaved Caspase-3	4.86E-06	5.31	5.68E-05	0.64	-0.65
cIAP-2	0.000593	3.23	0.001298	0.71#	-0.50
Clusterin	0.000961	3.02	0.001869	0.72#	-0.48
Bcl-2	0.00023	3.64	0.000805	0.72#	-0.47
Phospho-p53 (S15)	0.002082	2.68	0.003644	1.36#	0.45
HO-1/HMOX1/HSP32	0.001036	2.98	0.001908	1.42	0.50
Livin?	0.000371	3.43	0.000923	1.43	0.52
HO-2/HMOX2	0.000259	3.59	0.000824	1.51	0.59
p21/CIP1/CDKN1A	1.69E-05	4.77	0.000148	1.59	0.67
Fas/TNFRSF6/CD95	0.000783	3.11	0.001612	1.64	0.71
TRAIL R1/DR4	0.000115	3.94	0.000453	1.90	0.93
TRAIL R2/DR5	7.04E-08	7.15	2.46E-06	2.37	1.25
Phospho-p53 (S392)	0.000396	3.40	0.000923	3.81	1.93
Propolis vs control					
Bcl-x	9.42E-05	4.03	0.000389	0.28	-1.86
Claspin	0.000188	3.73	0.000689	0.41	-1.29
Pro-Caspase-3?	1.54E-05	4.81	0.00017	0.50	-1.00
TNF RI/TNFRSF1A	0.003504	2.46	0.007227	0.66	-0.60
Survivin	9.33E-05	4.03	0.000389	0.67	-0.58
Phospho-p53 (S392)?	0.012868	1.89	0.020221!	0.71#	-0.50
HIF-1 α	0.001659	2.78	0.004211	1.32#	0.41
HSP70	2.10E-05	4.68	0.000173	1.35#	0.43
FADD	4.36E-06	5.36	7.19E-05	1.40	0.48
HTRA2/Omi	0.000241	3.62	0.000796	1.66	0.73
Catalase	0.008252	2.08	0.013616!	1.68	0.74
TRAIL R1/DR4	0.000431	3.37	0.001292	1.89	0.92
TRAIL R2/DR5	3.03E-05	4.52	0.0002	2.30	1.20
HO-1/HMOX1/HSP32	1.64E-07	6.78	5.42E-06	2.84	1.51
Mixture vs (Doxorubicin and propolis)					
XIAP	0.001724	2.76	0.012071!	0.71#	-0.49

Dysregulated proteins* & pairwise comparisons	p value	-Log10(p)	FDR	Fold Change	log2(FC)
HSP60	0.006935	2.16	0.026969!	0.71#	-0.49
Cytochrome c	0.00385	2.41	0.016845!	0.72#	-0.47
HIF-1α	0.00381	2.42	0.016845!	0.77#	-0.38
cIAP-2	0.000396	3.40	0.00462	1.50	0.58
p27/Kip1	0.001031	2.99	0.009018	1.50	0.58
Claspin	0.000272	3.57	0.00462	1.62	0.69
PON2	3.11E-08	7.51	1.09E-06	1.88	0.91
Catalase	0.002956	2.53	0.016845!	2.04	1.03

*red and blue coloured font of the proteins is significantly upregulated and downregulated respectively in its corresponding pairwise comparison. #; Slightly above the threshold for protein filtration,!; FDR>0.01.

Table S6 Reactome overrepresented pathways using dysregulated proteins in MCF-7 cells treated with the synergistic propolis and doxorubicin combination

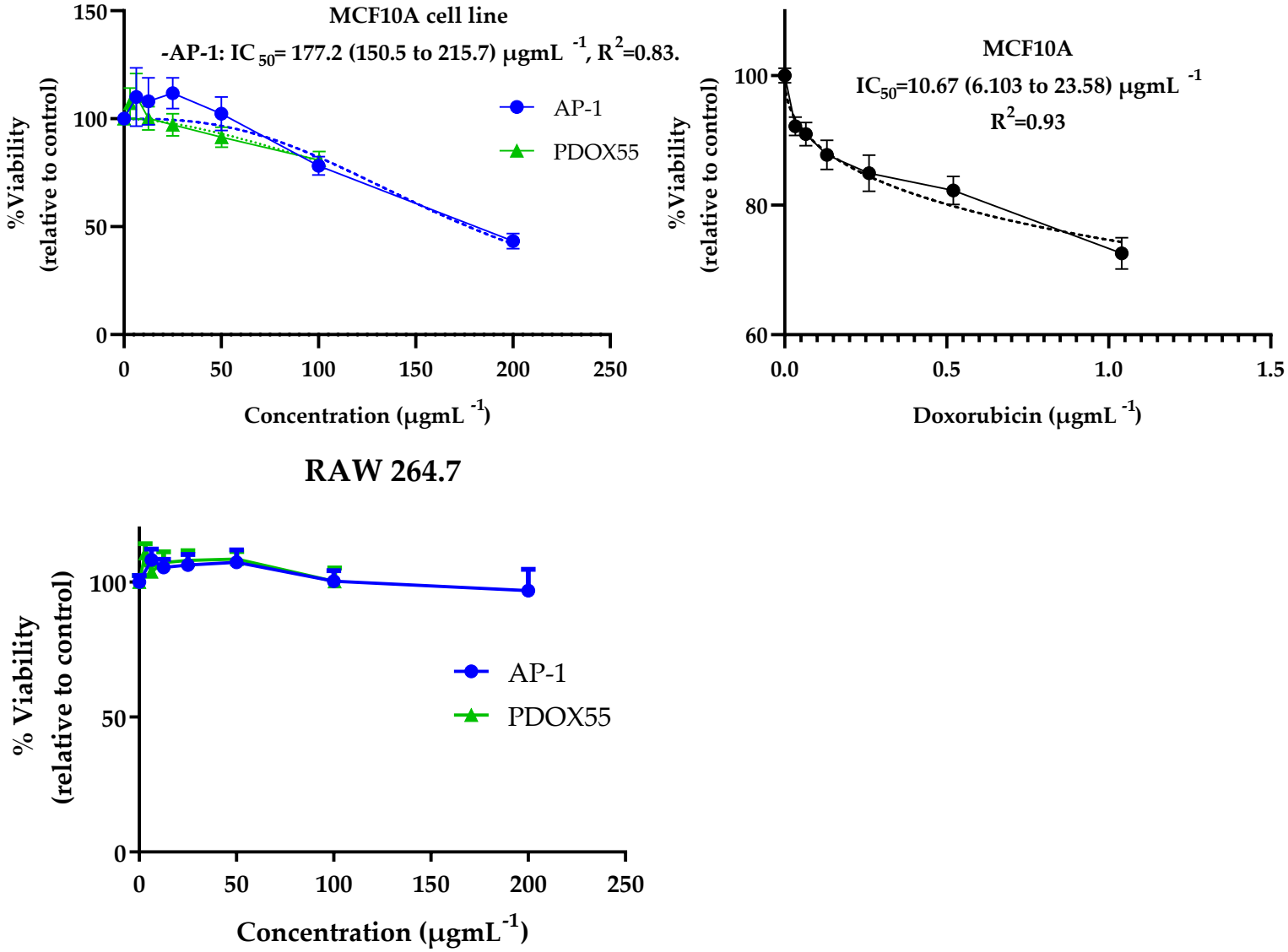
Pathway name	#Entities found	#Entities total	Entities pValue	Entities FDR	Rx found	Rx total	Rx ratio	Submitted entities found
TP53 Regulates Transcription of DNA Repair Genes	3	89	1.88E-05	0.001636	3	17	0.001266	BRCA1
Transcriptional Regulation by E2F6	2	46	3.56E-04	0.015304	2	33	0.002458	BRCA1
Ion transport by P-type ATPases	2	71	8.41E-04	0.017343	2	15	0.001117	ATP8B4
Diseases of DNA Double-Strand Break Repair	1	2	0.001239	0.017343	2	2	1.49E-04	BRCA1
Defective DNA double strand break response due to BRCA1 loss of function	1	2	0.001239	0.017343	1	1	7.45E-05	BRCA1
Transcriptional Regulation by TP53	3	487	0.002721	0.032654	4	259	0.019291	BRCA1
TP53 Regulates Transcription of DNA Repair Genes	3	89	2.07E-04	0.022271	3	17	0.001266	MDC1
Transcriptional Regulation by TP53	5	487	3.32E-04	0.022271	5	259	0.019291	MDC1;CHD3; COX6B1

*Upregulated and downregulated proteins are red and blue coloured, respectively.

Table S7 IMPala overrepresented pathways using dysregulated proteins in MCF-7 cells treated with the synergistic propolis and doxorubicin combination

pathway_name	pathway_source	genes	overlapping_genes	num_all_pathway_genes	P_genes	Q_genes
brca1 dependent ub ligase activity	BioCarta	1	BRCA1	8 (8)	0.00308	1
Transcriptional Regulation by E2F6	Wikipathways	1	BRCA1	10 (10)	0.00384	1
TP53 Regulates Transcription of DNA Repair Genes	Wikipathways	1	BRCA1	14 (14)	0.00538	1
PI3K-Akt Signaling Pathway	Wikipathways	2	BRCA1;LAMA4	339 (340)	0.00644	1
atm signaling pathway	BioCarta	1	BRCA1	18 (18)	0.00691	1
MET activates PTK2 signaling	Reactome	1	LAMA4	18 (18)	0.00691	1
PI3K-Akt signaling pathway - Homo sapiens (human)	KEGG	2	BRCA1;LAMA4	353 (354)	0.00697	1
prion pathway	BioCarta	1	LAMA4	19 (19)	0.00729	1
cell cycle: g2/m checkpoint	BioCarta	1	BRCA1	21 (21)	0.00806	1
role of brca1 brca2 and atr in cancer susceptibility	BioCarta	1	BRCA1	22 (22)	0.00844	1
Laminin interactions	Reactome	1	LAMA4	23 (23)	0.00882	1
Antigen Presentation: Folding_ assembly and peptide loading of class I MHC	Reactome	1	SEC23A	24 (24)	0.0092	1
ATM Signaling Network in Development and Disease	Wikipathways	2	MDC1;RIF1	46 (46)	0.000661	1
Nonhomologous End-Joining (NHEJ)	Reactome	2	MDC1;RIF1	69 (70)	0.00148	1
Transcriptional Regulation by TP53	Reactome	3	MDC1;CHD3;COX6B1	371 (374)	0.00321	1
SUMO E3 ligases SUMOylate target proteins	Reactome	2	MDC1;CHD3	120 (120)	0.00441	1
SUMOylation	Reactome	2	MDC1;CHD3	126 (126)	0.00485	1
DNA Double-Strand Break Repair	Reactome	2	MDC1;RIF1	167 (169)	0.00837	1

*Upregulated and downregulated proteins are red and blue coloured, respectively.



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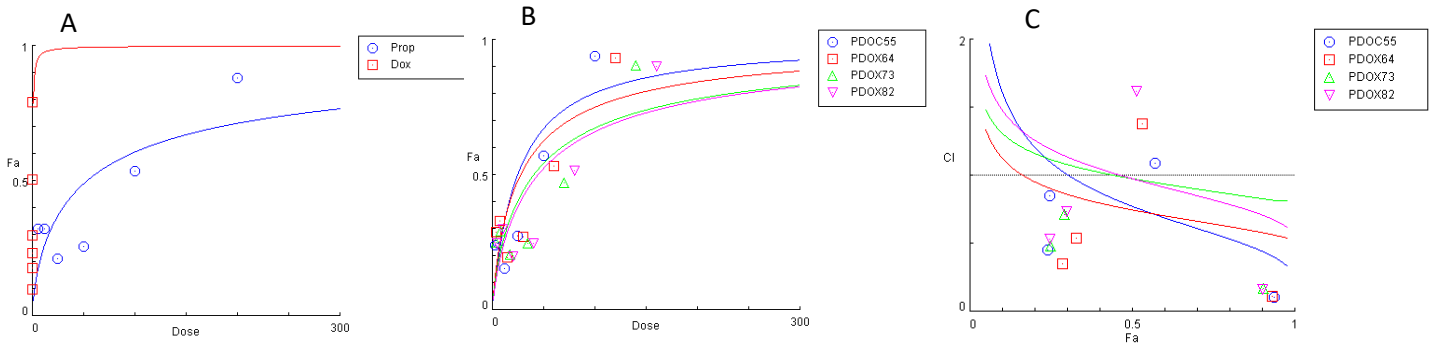
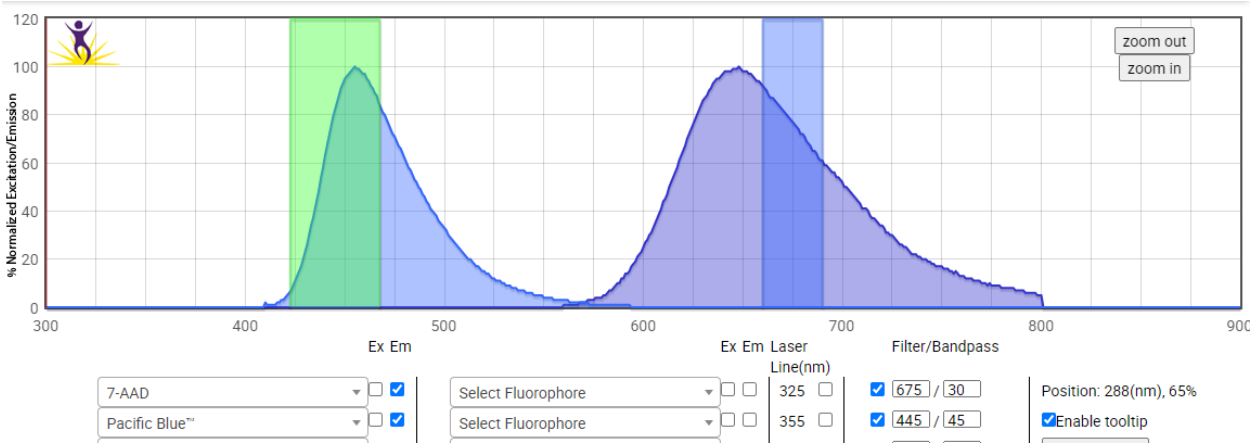


Figure S2 Dose-response curves of (A) Australian propolis (Prop), doxorubicin (Dox) and most synergistic combinations (B) together with its combination index (CI) plot (C) calculated from CompuSyn software.



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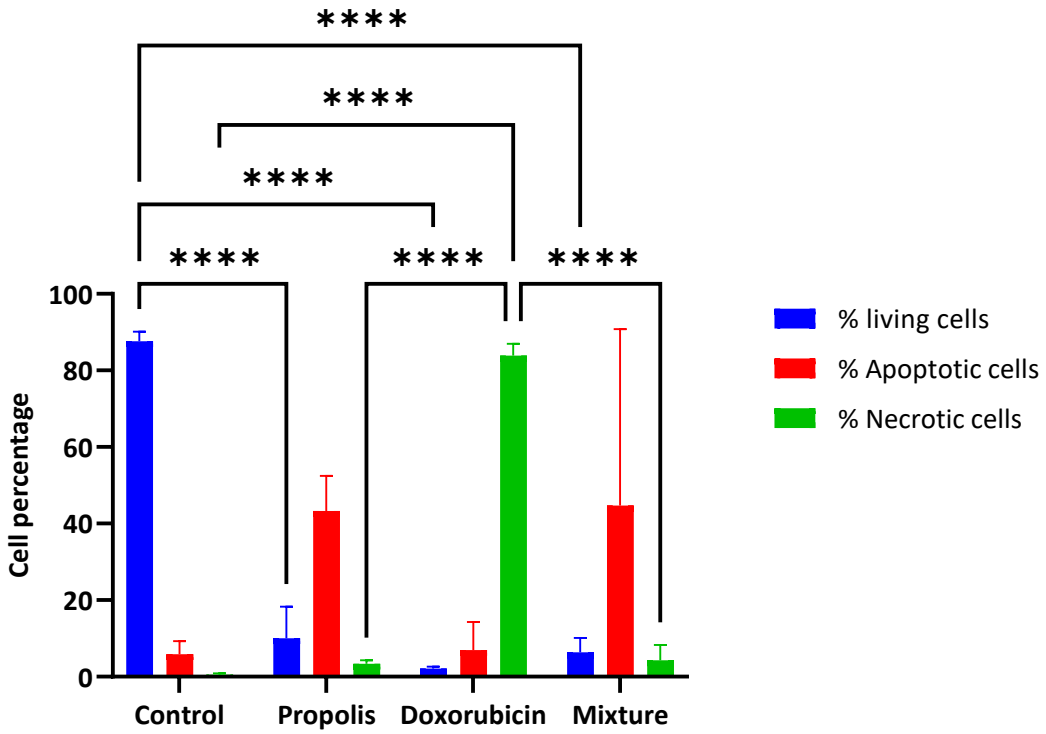


Figure S4 Combined cell percentage analysis in different treatment groups in quadruplicates. Twenty-four hours treatment of propolis extract (100 µgmL^{-1}), Dox; doxorubicin (0.22 µgmL^{-1}), and their mixture at a half-dose synergistic combination (50 µgmL^{-1} and 0.11 µgmL^{-1}, respectively) with a negative control were implemented using antibodies against Annexin-V CF-Blue and the reporter 7AAD. **; significantly different as derived from Two-way ANOVA and Tukey's multiple comparisons at P<0.001, n=4.**

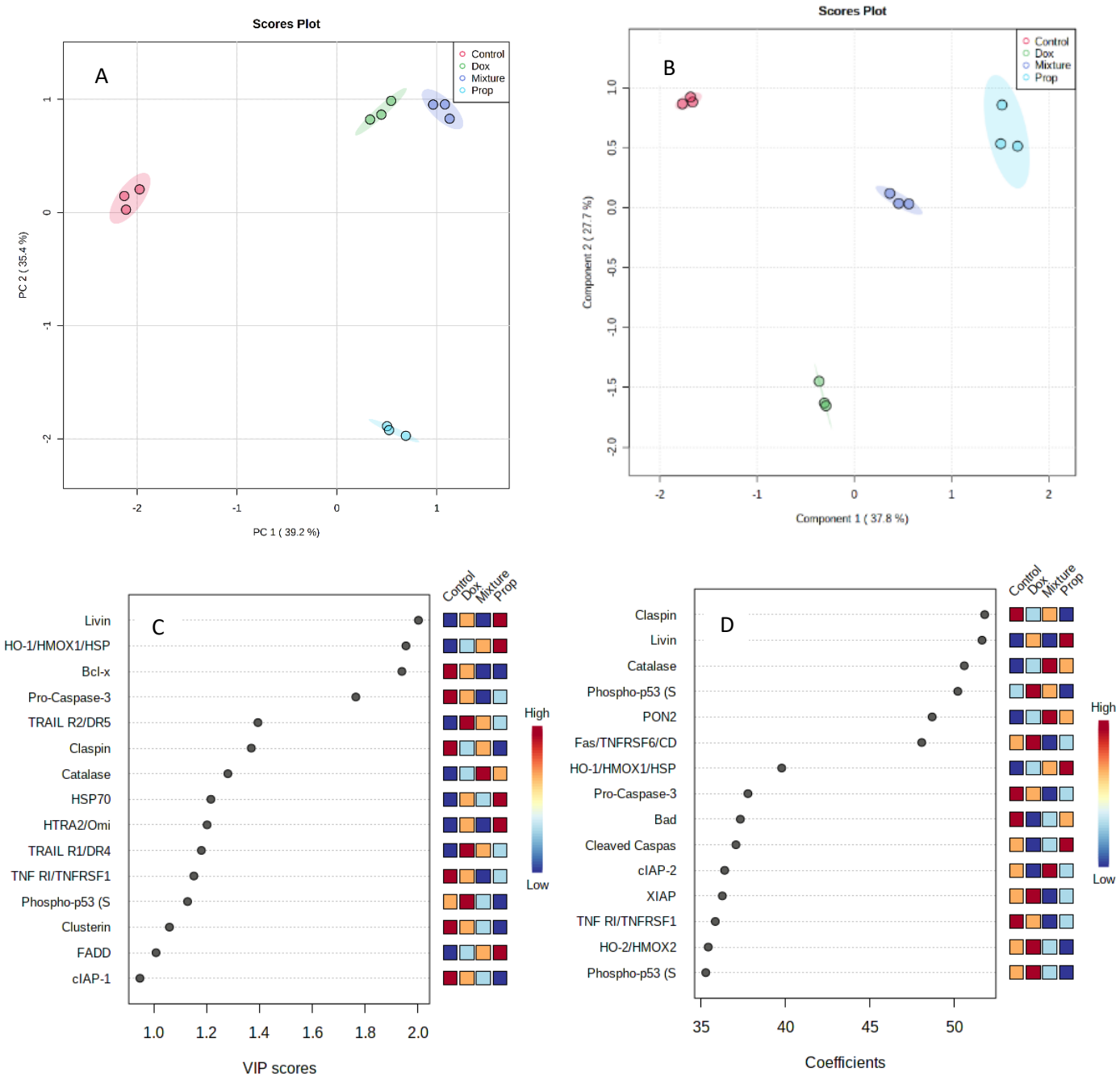
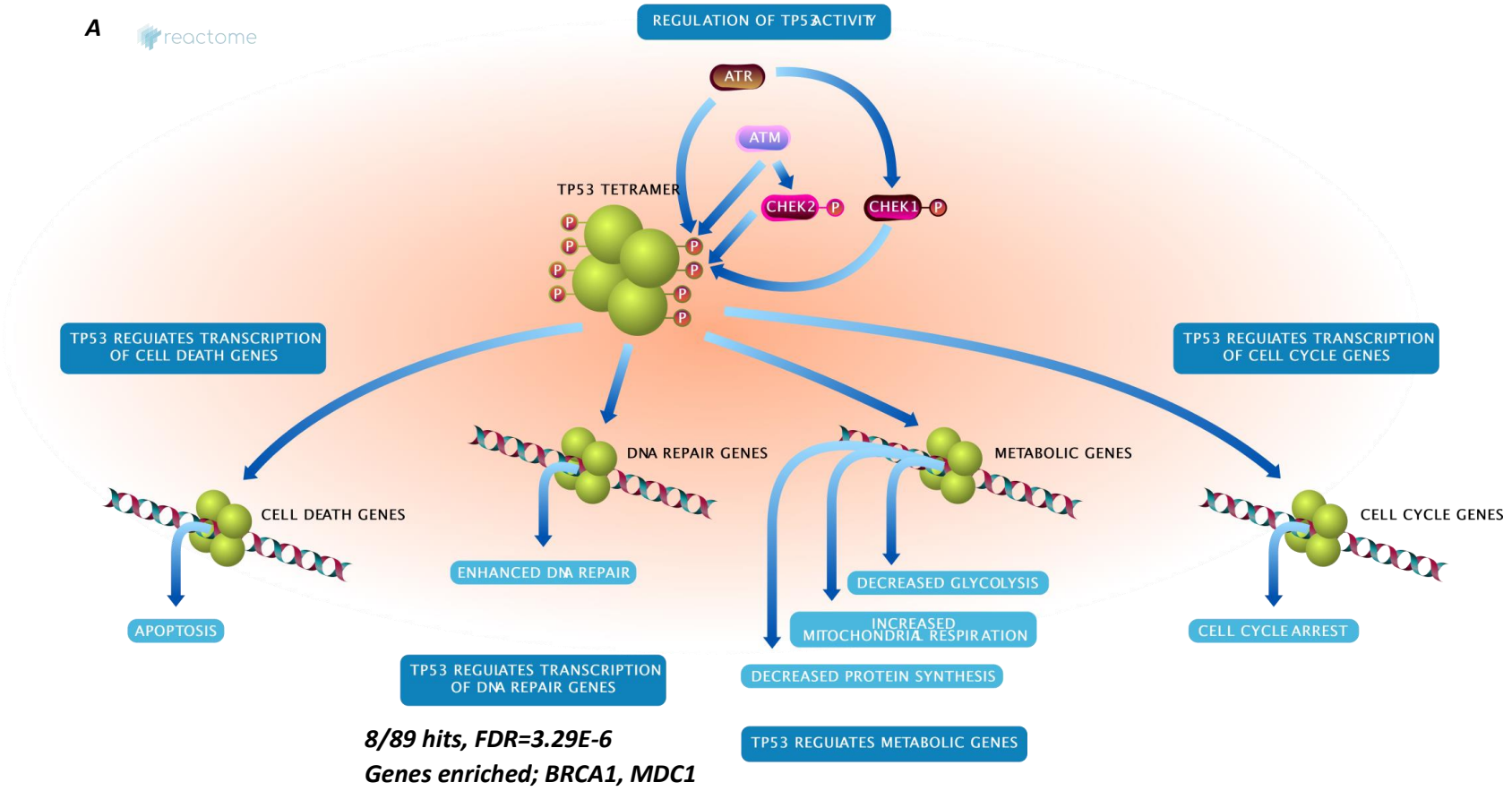


Figure S5 Supervised PLS-DA and Unsupervised PCA multivariate data analysis of the apoptotic proteins of MCF-7 lysates analysed by Proteome profiler human apoptotic array kit. (A) Score plot of principal component analysis (PCA) between the selected principal components alongside the explained variances shown in brackets. (B) Score plot of partial least square discriminant analysis (PLS-DA) where third averaged point was imputed for each treatment, (C and D) are the most discriminatory proteins in among different treatments identified by variable importance projection (VIP) score and coefficients of PLS-Da model, respectively. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.



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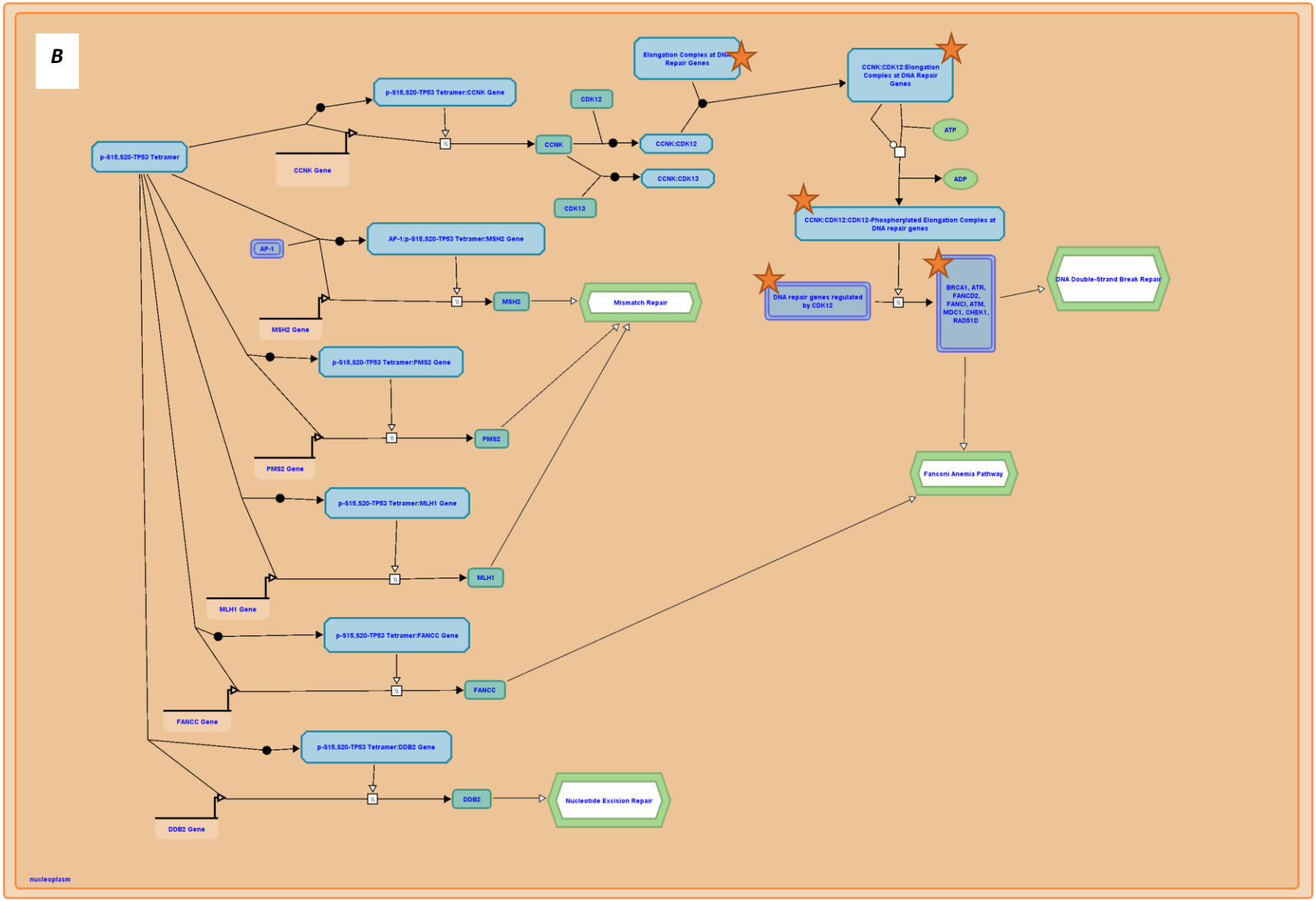

Figure S6 continued

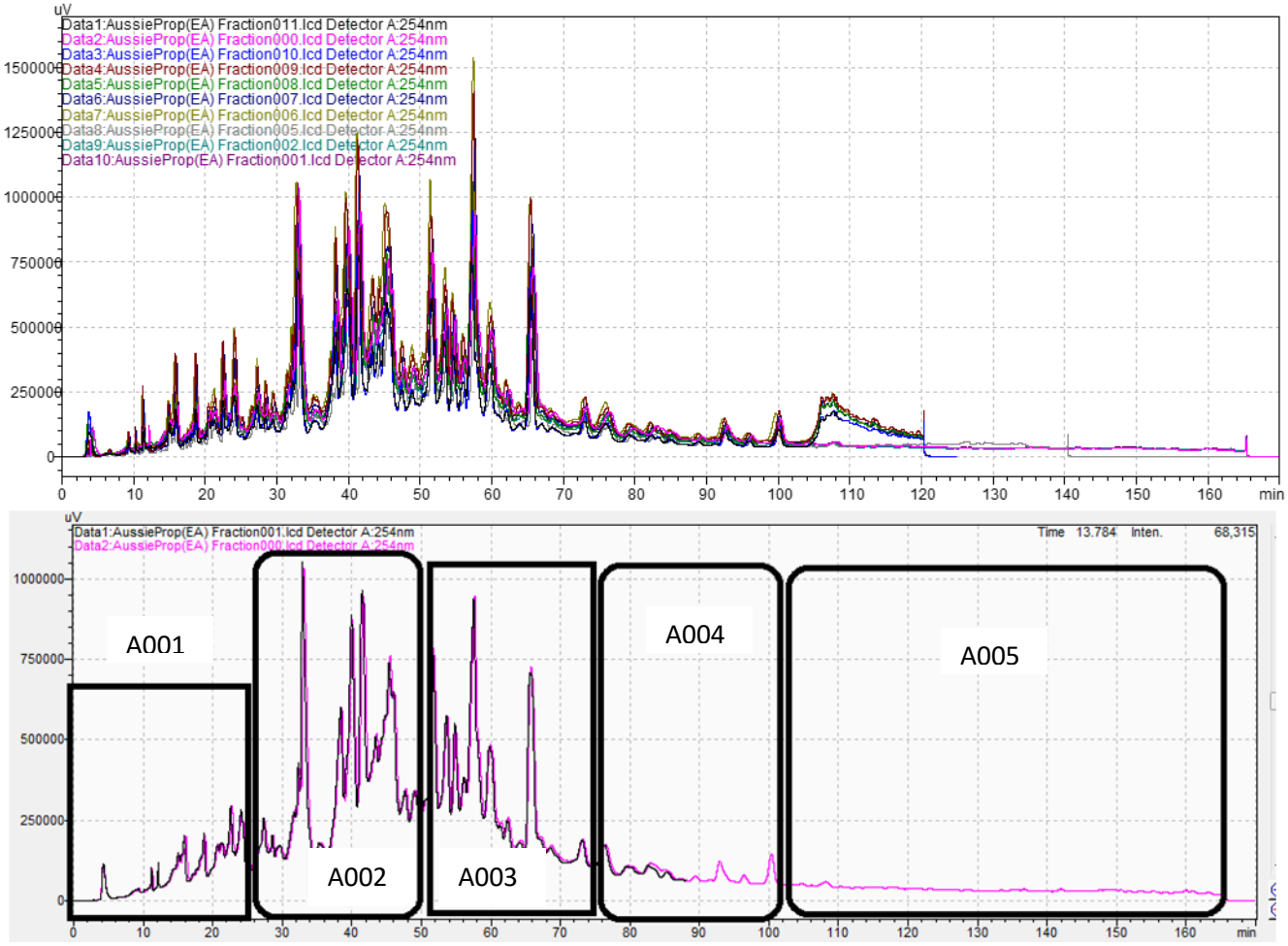

Figure S7 Preparative HPLC fractionation of Australian propolis extract