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Supplementary file of:

Novel pH-sensitive and biodegradable micelles for combination delivery of Doxorubicin and Conferone to induce apoptosis in MDA-MB-231 breast cancer cell line

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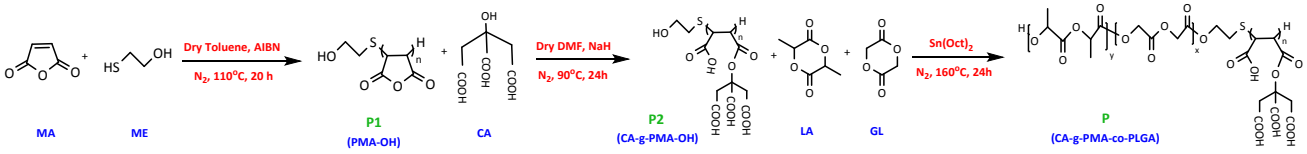


*Figure 1-S: synthesis root of copolymer, Step 1: Synthesis of hydroxy terminated poly maleic anhydride (***P1***: PMA-OH), Step 2: Functionalization of PMA-OH with citric acid (***P2***: CA-g-PMA-OH), Step 3: Post co-polymerization of CA-g-PMA-OH with Lactide and Glycolide (***P***: CA-g-PMA-co-PLGA).*

**This figure is the same with Figure 1 in paper but in *.cdx file type.*

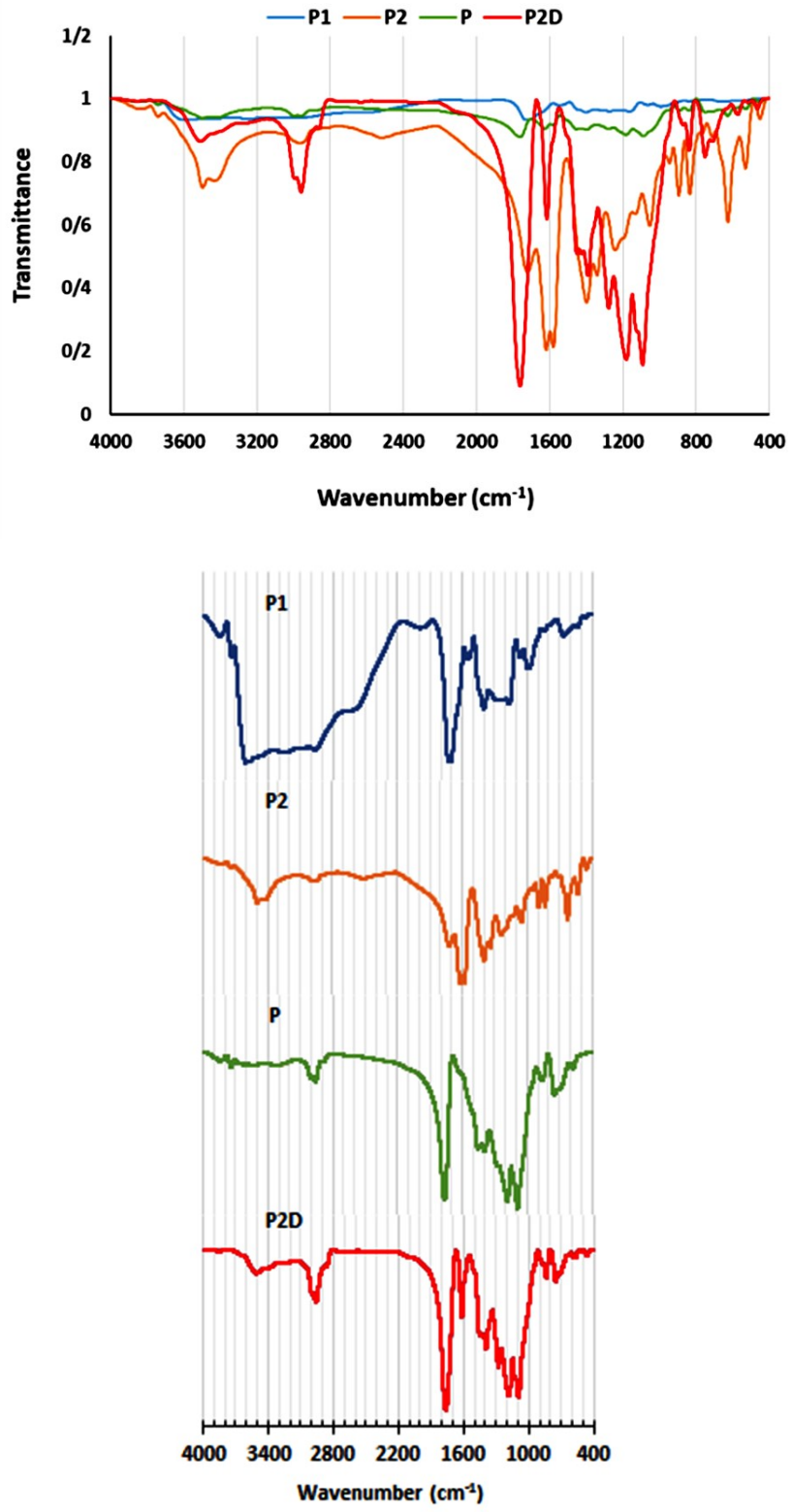


Figure 2-S: FTIR Spectra of: ***P1****: (PMA-OH),* ***P2****: (CA-g-PMA-OH),* ***P****: (CA-g-PMA-co-PLGA),* ***P2D****: (Co-drug loaded copolymeric micelles).*

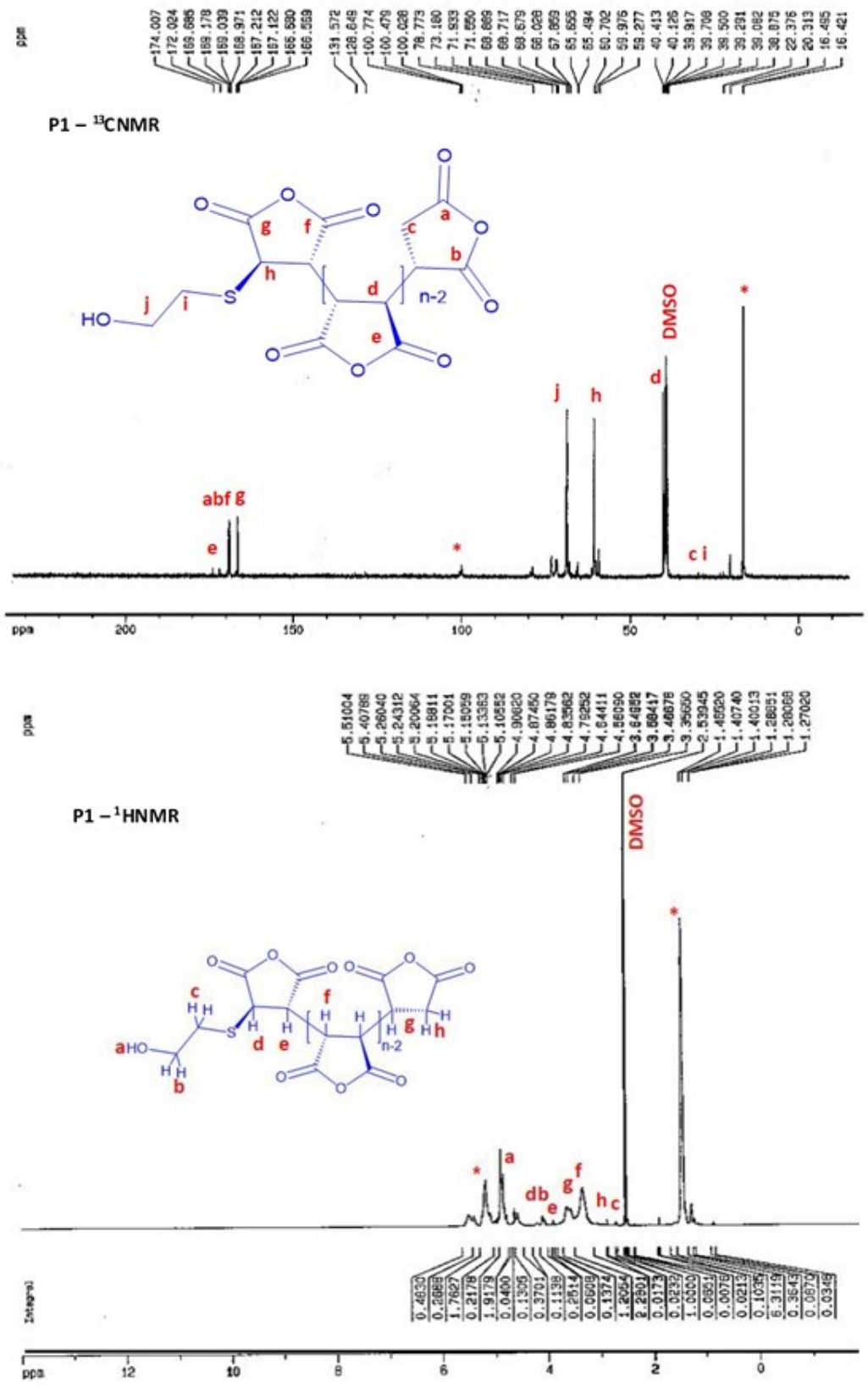


*Figure 3-S: NMR spectra of **P1**: **^{13}CNMR** spectra, that * are the peaks related to solvent residu**e and ^{1}HNMR** Spectra of **P1**, that * are the peaks related to free mercapto ethanol**e.***

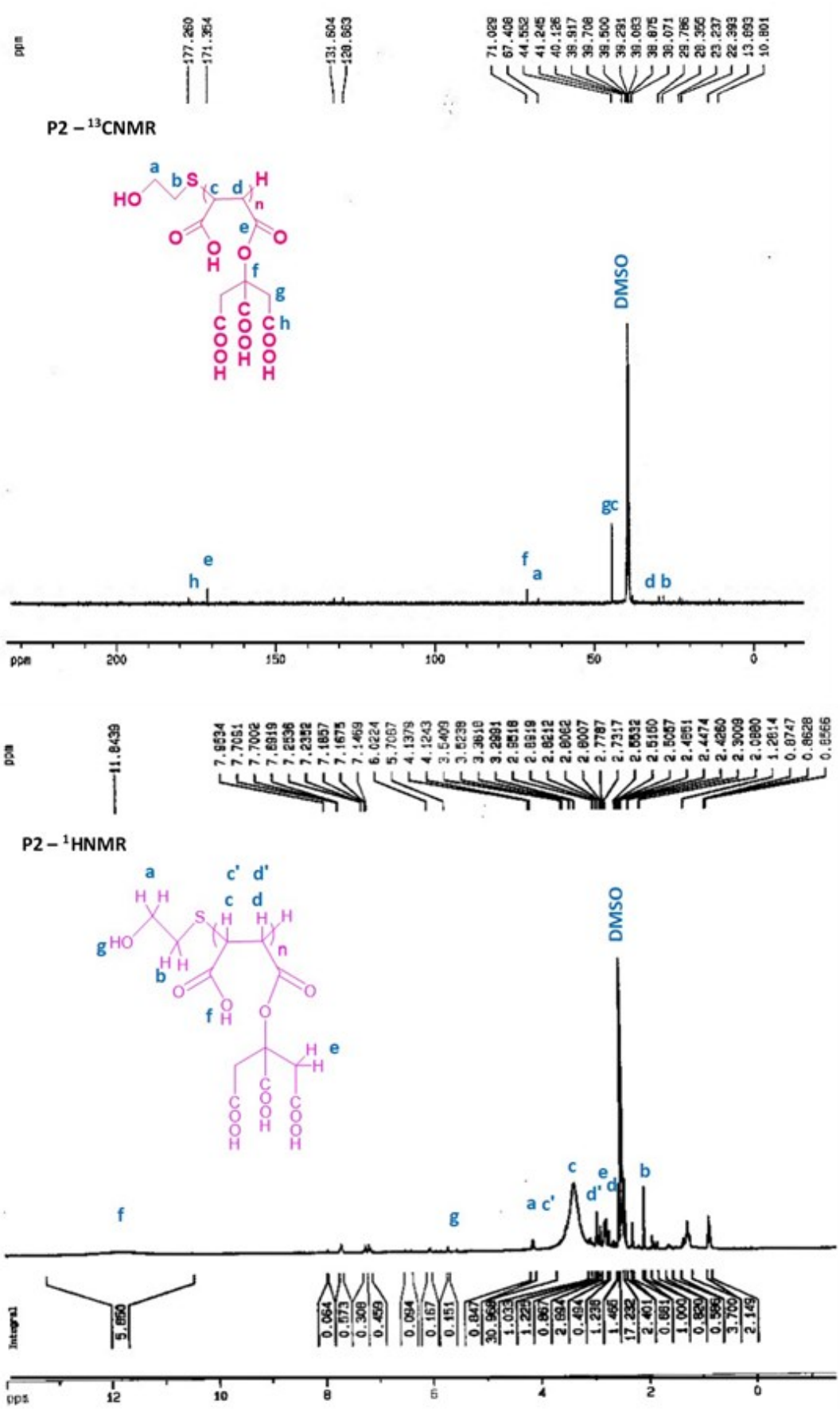


*Figure 4-S: NMR spectra of **P2**: **^{13}CNMR** spectra of **P2**, **and ^{1}HNMR** Spectra of **P2**, that c' and d' are the peaks related to the signals pertained to maleate group that was banded to -S.*

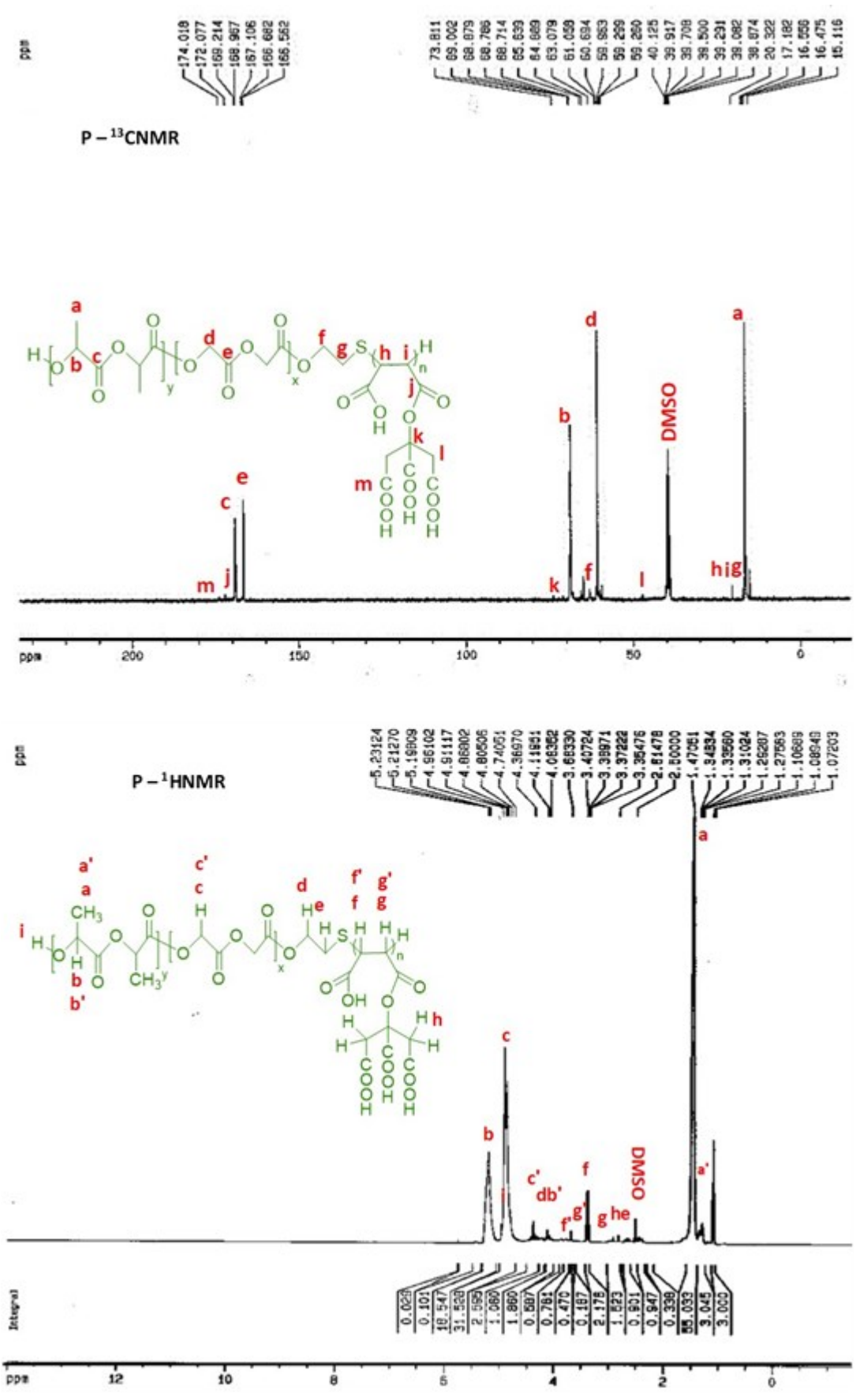


*Figure 5-S: NMR spectra of **P**: **^{13}CNMR** spectra of **P, and ^{1}HNMR** Spectra of **P**, that a', b' and c' are the peaks related to end groups, and f' and g'are the signals pertained to maleate group that was banded to -S.*

Calculation of molar mass of copolymer by ^{1}HNMR spectra

The molar mass (M_n) of copolymer was determined with ^{1}HNMR spectra, by integrating the signals pertaining to each monomer using following equation [Jay Wm. Wackerly, J. Chem. Educ]:

$$n_{polymer} = \frac{\sum_{i=1}^{m} \frac{I_i}{p_i}}{m}$$

$M_n = n.\,(monomers\ molecular\ mass)$

Where, *m* is the number of polymer signals that used, I_i and p_i are the integration and number of protons related to *i*th polymer signal.

$$n_{Lactide} = \frac{\frac{55.03}{6} + \frac{18.542}{2}}{2} = 9.22$$

$$n_{Glycolide} = \frac{\frac{31.528}{4}}{1} = 7.882$$

$$n_{MA} = \frac{\frac{2.176}{1}}{1} = 2.176$$

$$n_{CA} = \frac{\frac{0.901}{4}}{1} = 0.225$$

$$n_{ME} = \frac{\frac{1.0596}{2}}{1} = 0.53$$

$$n_{GL\ end\ group} = \frac{\frac{2.5951}{2}}{1} = 1.297$$

$$n_{LA\ end\ group} = \frac{\frac{1.86}{1}}{1} = 1.86$$

$$M_n = (9.22 \times 144.13) + (7.882 \times 116.07) + (2.176 \times 98.06) + (0.225 \times 210.14) + (0.53 \times 7$$
$$+ (1.86 \times 144.13) + 1 = 2965.43\ g/mol$$

$$M_n = (9.22 \times 144.13) + (7.882 \times 116.07) + (2.176 \times 98.06) + (0.225 \times 210.14) + (0.53 \times 7$$
$$+ 1 = 2670.717\ g/mol$$

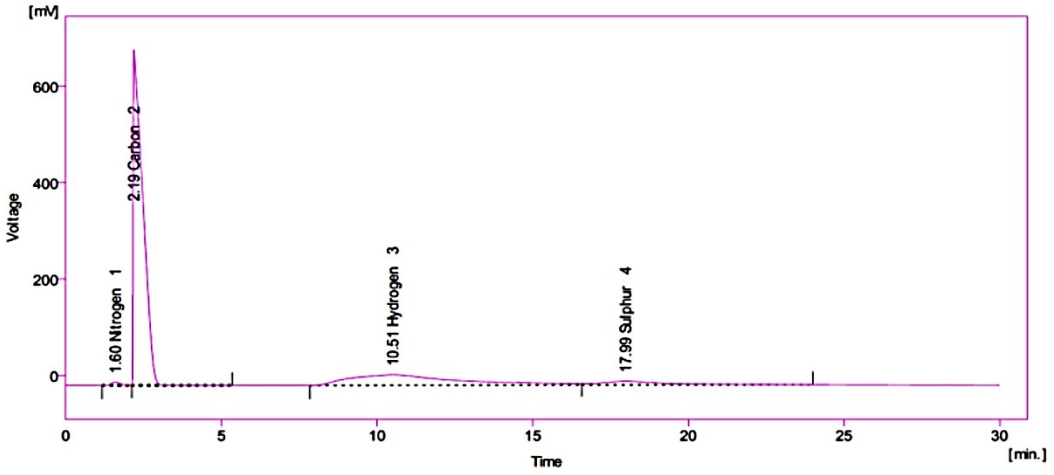


Figure 6-S: CHNS elemental analysis: plot of time (min) versus voltage (mV)

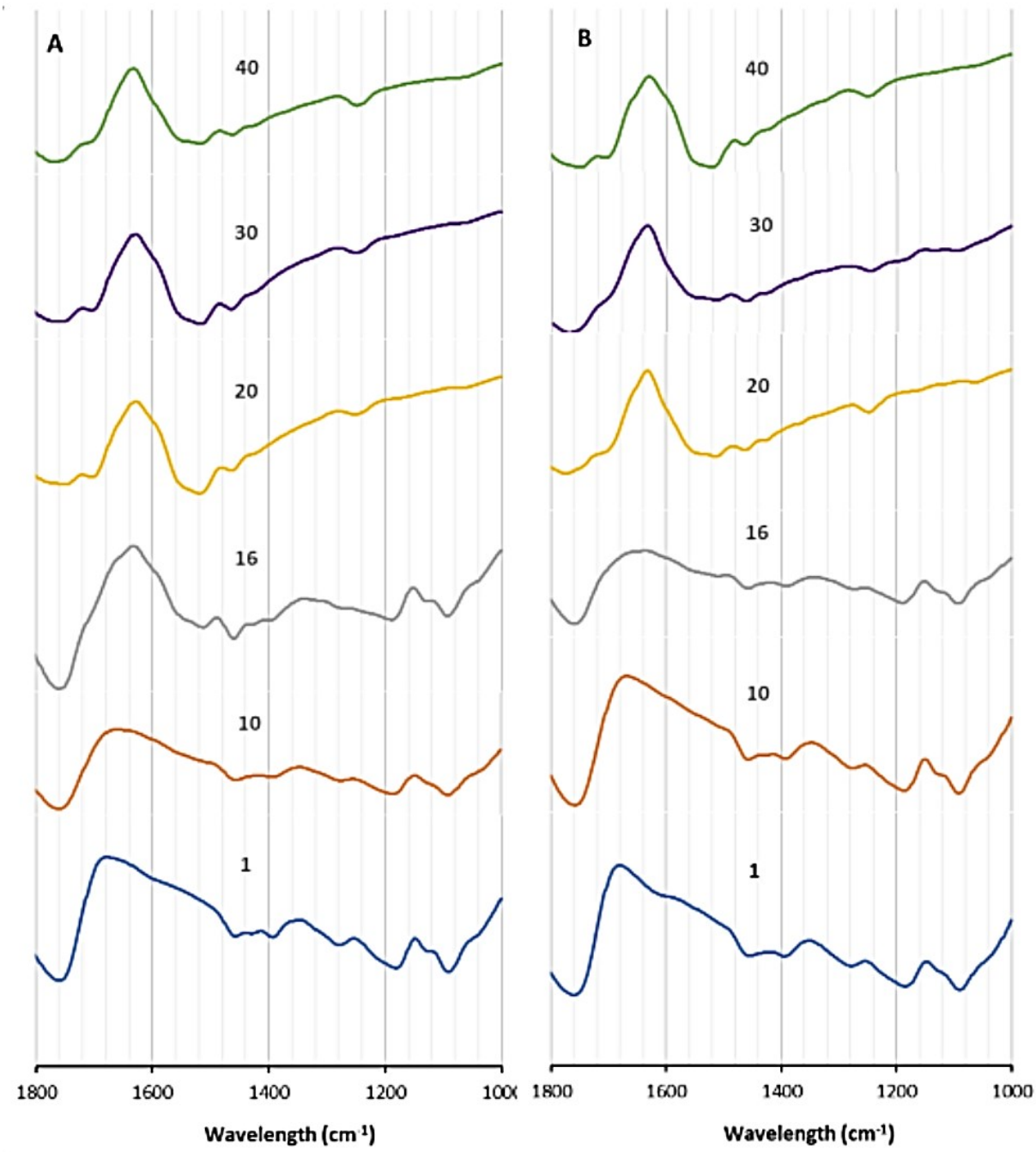
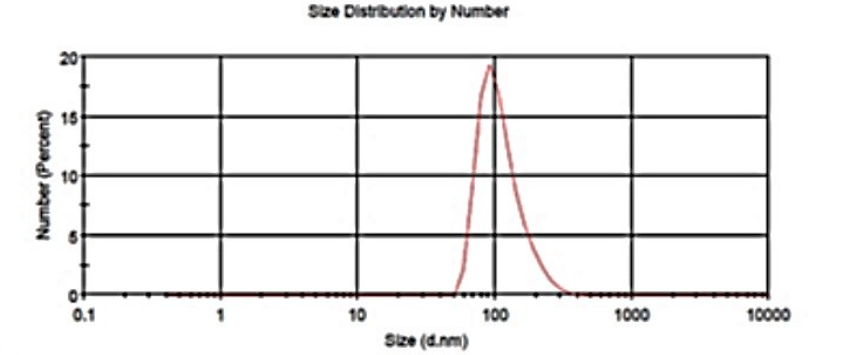


Figure 7-S: Copolymer FTIR spectra during degradation test after 1, 10, 16, 20, 30 and 40 day, wavenumber between 1000-1800 cm^{-1}, in PBS with pH value of A) pH = 5.5; B) pH = 7.4.

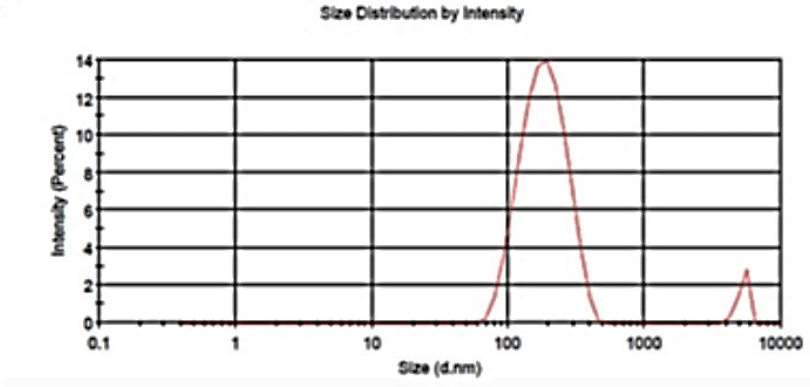
A

		Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm): 192.6	Peak 1:	112.6	100.0	44.13
PdI: 0.367	Peak 2:	0.000	0.0	0.000
Intercept: 0.930	Peak 3:	0.000	0.0	0.000

Result quality Refer to quality report



B



C

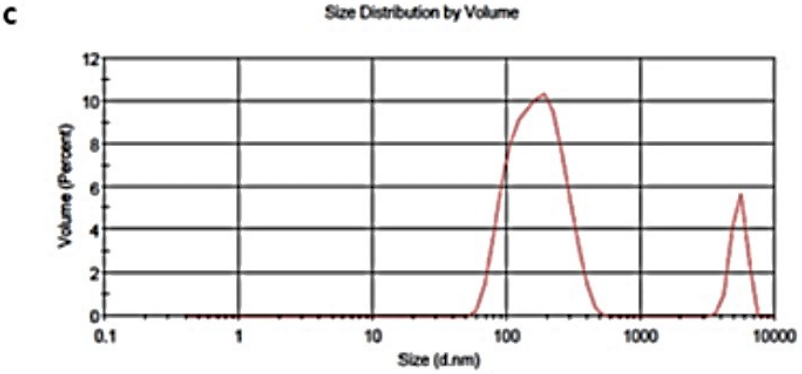
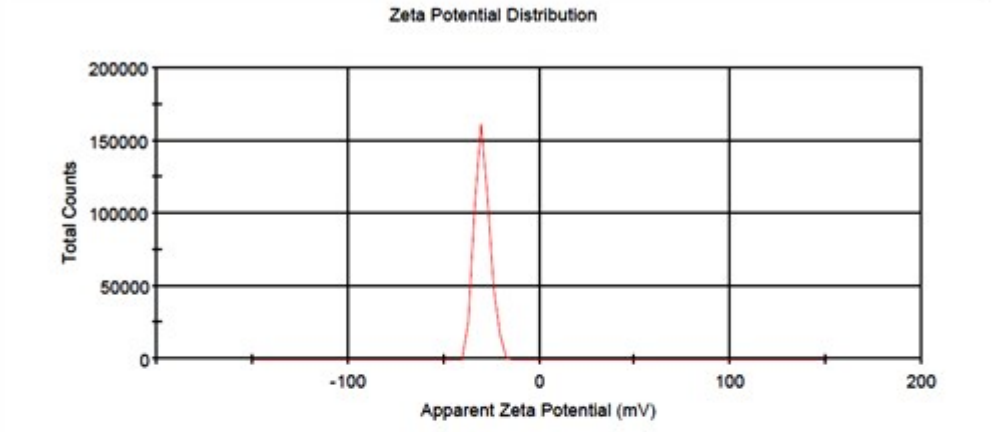


Figure 8-S: DLS results of P, A) number percent versus size (nm); B) Intensity percent versus size (nm); C) Volume percent versus size (nm).

A

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -29.7	Peak 1:	-29.7	100.0	3.93
Zeta Deviation (mV): 3.93	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.00830	Peak 3:	0.00	0.0	0.00
Result quality Good				



B

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -6.57	Peak 1:	-6.57	100.0	3.61
Zeta Deviation (mV): 3.61	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.216	Peak 3:	0.00	0.0	0.00
Result quality Good				

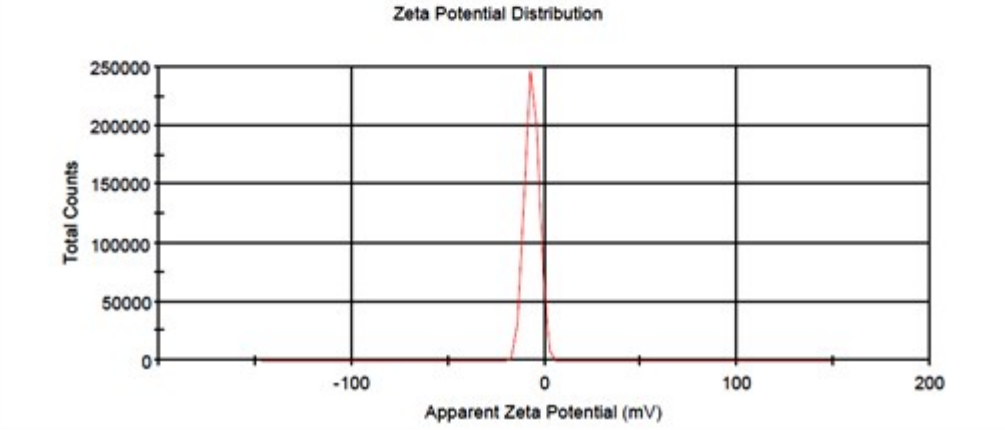


*Figure 9-S: Results of zeta potential (mV) measurments for: A) Blank micelles (**P**) ; B) Co-drug loaded micelles (**P2D**)*

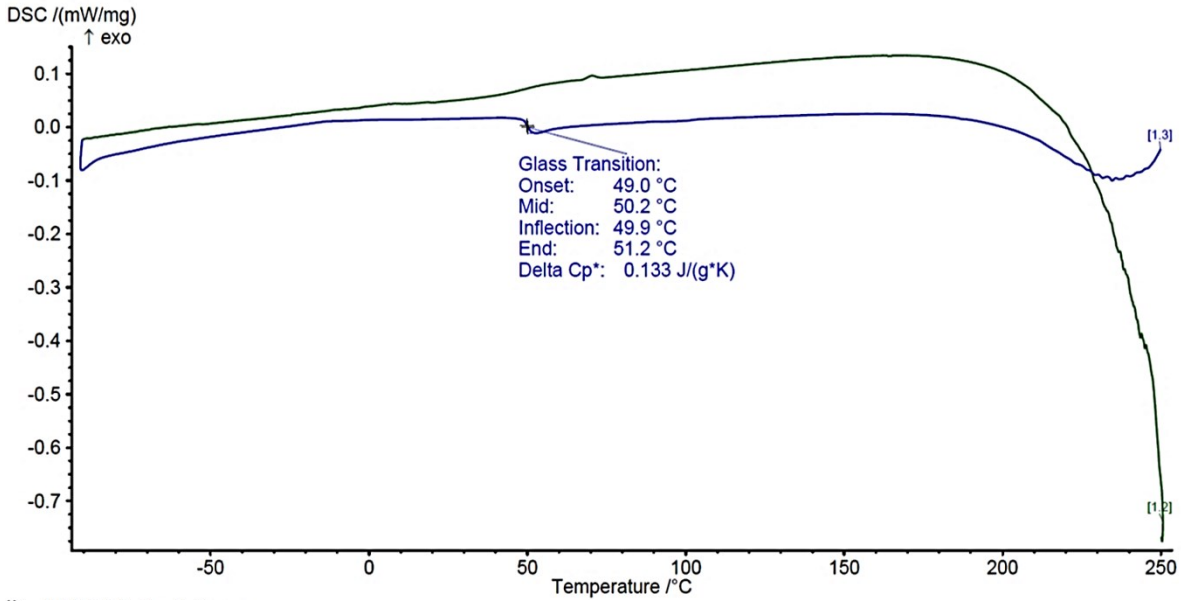


Figure 10-S: DSC plot of copolymer, plot of temperature vs. DSC.

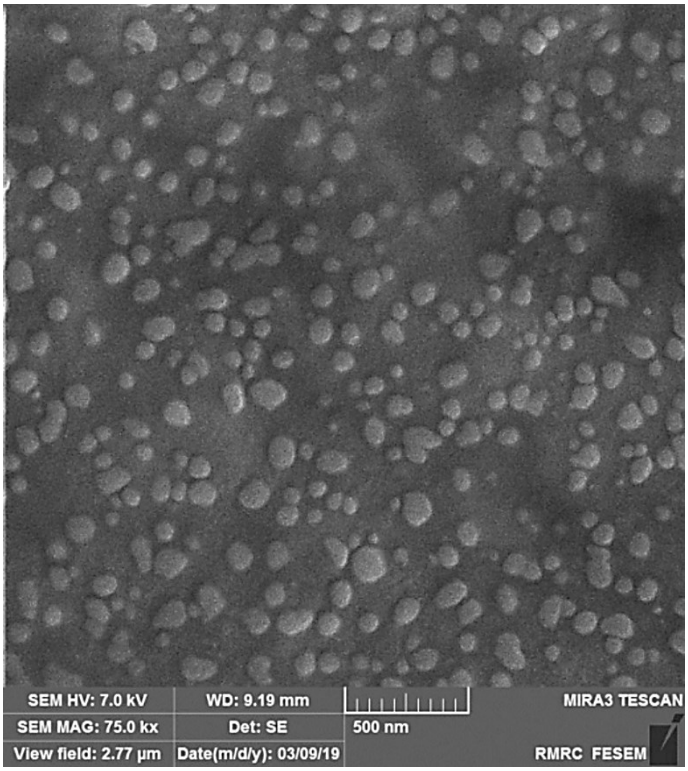


*Figure 11-S: SEM image of blank micelles (**P**)*

Table 1-S: Percentage of cellular uptake of RB-P and RB-P2D micelles in 0.5, 1.5, and 3 h into MDA-MB-231 cell line using flowcytometery.

Cellular uptake %	Time (h)		
	0.5	**1.5**	**3**
P	33.2	60.5	81.5
P2D	97.0	99.7	100

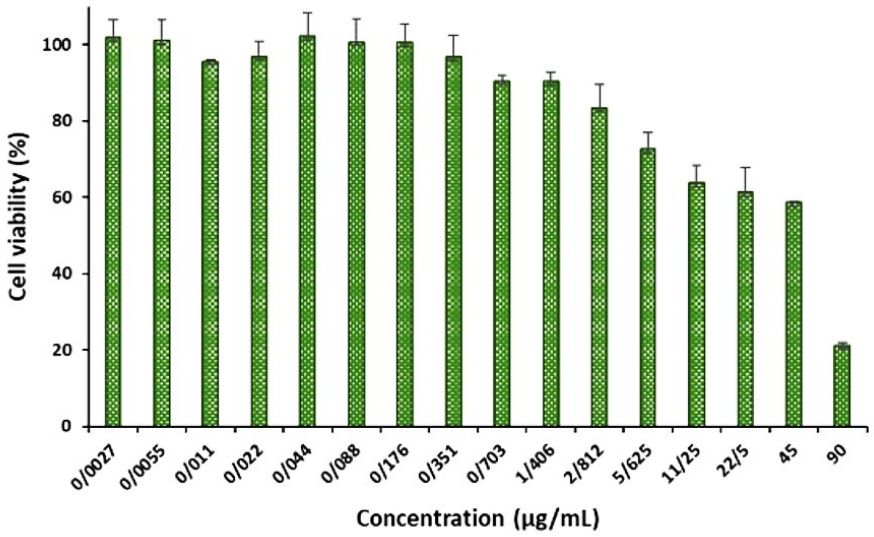


Figure 12-S: Conferone cytotoxicity on MDA-MB-231 cell lines, by MTT method.

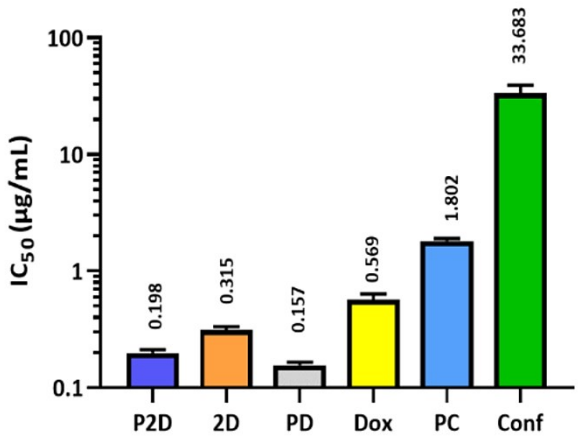


Figure 13-S: IC_{50} plot of all formulations, P2D, 2D, PD, Dox, PC and Conf; calculated by prism software (P < 0.0001).

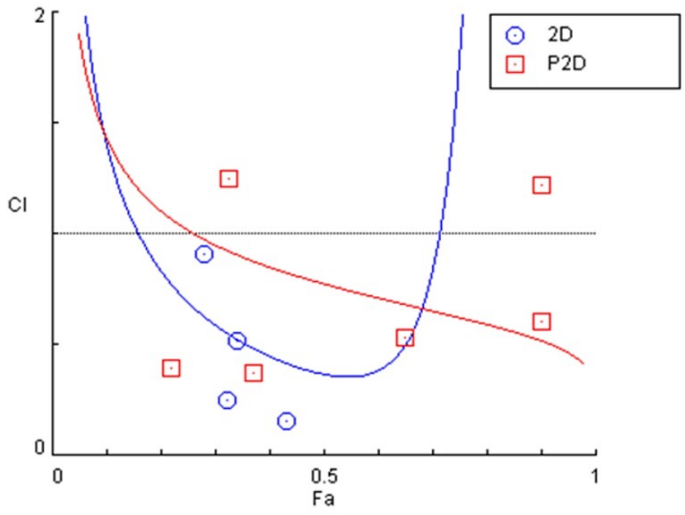


Figure 14-S: Combination Index (CI) plot, for P2D and 2D.

Table 2-S: Combination index (CI) results for P2D and 2D, in different doses, calculated by CompuSyn software.

Total dose (ppm)	P2D		2D	
	Fa	CI	Fa	CI
0.058	0.9	0.605	0.873	20.054
0.117	0.9	1.221	0.891	93.898
0.234	0.65	0.533	0.946	7739.77
0.468	0.37	0.370	0.431	0.156
0.937	0.221	0.393	0.324	0.249
1.875	0.326	1.25	0.341	0.518
3.75	0.284	2.101	0.282	0.909

Table 3-S: Cell cycle results of formulation on MDA-MB 231 cell line using flowcytometery.

formulations	Cell cycles (%)			
	Sub G_0	G_0/G_1	S	G_2/M
P2D	12.84	20.75	23.60	33.63
2D	15.63	53.88	17.91	11.25
PD	12.71	10.73	23.82	37.29
Dox	9.07	7.07	10.09	32.32
PC	1.29	10.03	8.05	38.93
Conf	3.61	59.84	18.27	16.40
P	3.78	46.31	11.45	29.83
Control	1.37	55.46	17.67	24.24

Table 4-S: Results of apoptosis of MDA-MB-231 cell line treated with formulation (P2D, 2D, PD, Dox, PC and Conf) using flowcytometery

Cell %	Viable	Early apoptosis	Late apoptosis	Necrosis
P2D	0.95	1.45	93.9	3.72
2D	12.2	22.6	36.1	29.2
PD	7.17	4.89	74.9	13.0
Dox	51.7	17.9	8.27	22.1
PC	45.4	12.0	37.8	4.77
Conf	81.4	3.97	14.1	0.55
Control	99.5	0.0	0.0	0.50

Table 5-S: Western blotting results of P2D, fold change of Bax, Bcl2, pro-Casp9, Cleaved-Casp9, pro-Casp3, Cleaved-Casp3, pro-Casp7, Cleaved-Casp7 and P27 proteins related to control (= 1).

Fold change	proteins								
	Bcl-2	**Bax**	**Pro-casp9**	**Cl-casp9**	**Pro-casp3**	**Cl-casp3**	**Pro-casp7**	**Cl-casp7**	**P27**
P2D	0.839	1.7	0.634	1.76	0.89	1.82	0.696	1.033	1.04