Chapter IV

Results

4.1 Hemolysis assay of microemulsion formulations

The hemolysis assay was employed to assess the damage of red blood cells caused by 10μM of the formulations of blank microemulsions (MEa10, MEb10 and MEc10), gemcitabine—loaded microemulsions (MEa10+, MEb10+ and MEc10+) and gemcitabine solution (GEM10). As shown in Table 4.1 and illustrated in Figure 4.1, GEM10 has the largest effect on the red blood cells by revealing the maximum percentage of hemolysis activity of 36.77 ± 4.20. The blank microemulsion formulations, MEb10 and MEc10, have hemolytic effects of 9.72 ± 2.47 and 7.18 ± 1.93, respectively, which are less than the hemolytic effect of MEb10+ and MEc10+ that have percentages of 17.27 ± 0.14 and 21.37 ± 3.70, respectively. However, MEa10 and MEa10+ showed similar percentages of hemolytic effect of 21.38 ± 3.49 and 21.53 ± 3.41, respectively. It is worth noting that of the gemcitabine-loaded microemulsions, MEb10+ has the smallest effect on the red blood cells by showing percentages of hemolysis activity of 17.27 ± 0.14.

Table 1.1 The percentages of hemolysis activity of different formulations of 10μM of blank microemulsions (MEa10, MEb10, and MEc10), gemcitabine-

loaded microemulsions (MEa10+, MEb10+ and MEc10+) and gemcitabine solution (GEM10), expressed as $\bar{X}$± SD.

Formulations	% Hemolysis Activity	% CV*
MEa10	21.38± 3.49	16.32
MEa10+	21.53 ± 3.41	15.83
MEb10	9.72 ± 2.47	25.41
MEb10+	17.27 ± 0.14	0.81
MEc10	7.18 ± 1.93	26.88
MEc10+	21.37 ± 3.70	17.31
Gemcitabine (GEM10)	36.77 ± 4.20	11.42

*CV is the coefficient of variation measured through dividing the standard deviation by the mean.

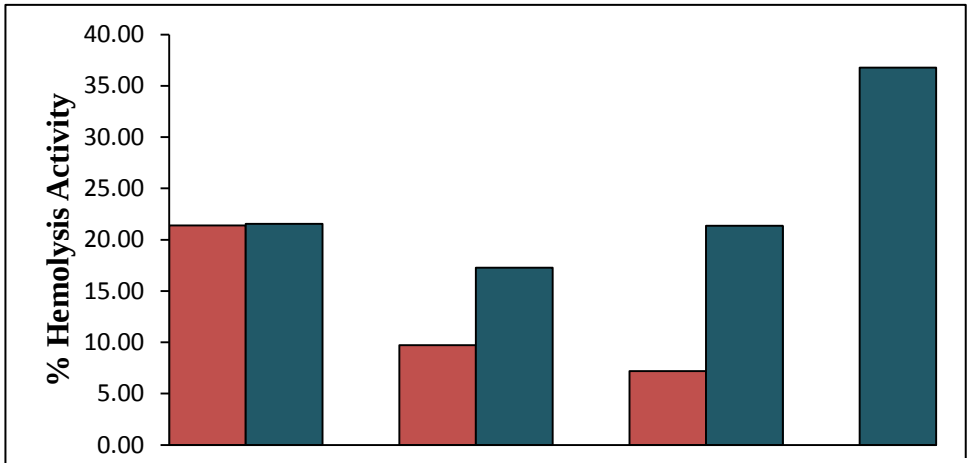


Figure 4.1 The percentages of hemolysis activity of different formulations of 10μM of blank microemulsions (MEa10, MEb10 and MEc10), gemcitabine-loaded microemulsions (MEa10+, MEb10+ and MEc10+) and gemcitabine solution (GEM10).

In order to assess the differences between the formulations used for the hemolysis assay, one-factor ANOVA and post hoc analyses which measure the *p*-

values for pairwise *t*-tests were implemented as demonstrated in Table 4.2. According to the one-factor ANOVA analyses, it has been found that there was a very highly significant difference between all of the microemulsion formulations and GEM10. The post hoc analyses revealed that there was a very highly significant difference between each microemulsion formulation either blank or drug-loaded and GEM10. The blank microemulsion formulations, MEb10 and MEc10, were having similar significant differences with all of the loaded microemulsion formulas (MEa10+, MEb10+ and MEc10+). In particular, both MEb10 and MEc10 were having very highly significant differences with MEa10+ and MEc10+ and a highly significant difference with MEb10+. In contrast, MEa10 did not have any significant difference with any drug-loaded microemulsion formula (MEa10+, MEb10+ and MEc10+). Among the blank microemulsion formulations, MEb10 and MEc10 did not have any significant difference between them, while there were very highly significant differences between the MEa10 and both MEb10 and MEc10.

Table 4.2 Statistical analyses of the percentages of hemolysis activity of 10 µM of different formulations of blank microemulsions (MEa10, MEb10 and MEc10), gemcitabine-loaded microemulsions (MEa10+, MEb10+ and MEc10+) and gemcitabine solution (GEM10) through identifying the *p*-value using one-factor ANOVA and post hoc analyses for pairwise t-tests.

	MEc10	**MEb10**	**MEb10+**	**MEc10+**	**MEa10**	**MEa10+**
MEc10						
MEb10	0.3258					
MEb10+	0.0012	0.0089				
MEc10+	0.0001	0.0004	0.1216			
MEa10	0.0001	0.0003	0.1202	0.9947		
MEa10+	4.87×10^{-05}	0.0003	0.1084	0.9475	0.9528	
GEM10	1.05×10^{-08}	3.28×10^{-08}	1.74×10^{-06}	2.35×10^{-05}	2.38×10^{-05}	2.64×10^{-05}

Note: Cells colored with dark pink have *p*-values with very highly significant differences between the groups while violet cells have highly significant differences among them.

4.2 Cytotoxicity screening using sulphorhodamine B assay

The anti-proliferative sulphorhodamine B (SRB) assay was employed to assess the growth inhibition and viability of the cells treated with the blank and drug-loaded microemulsion formulations. This colorimetric assay estimates the percentages of viable cells number indirectly by staining total cellular protein with the dye SRB. The high percentages of cell viability are an indication of low cytotoxicity of the formula, and vice versa. The human cells of A549, MCF-7, HCT-116 and HFS were incubated for 48 h in the culture media with 1 and 10 µM of blank microemulsions (MEa, MEb and MEc), gemcitabine loaded-microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM). It should be noted that all of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively.

As demonstrated in Table 4.3, the viability percentages of A549, MCF-7, HCT-116 and HFS cells varied by the administration of 1 µM of different microemulsion formulations and GEM1. The MEb1 was the most cytotoxic formula when subjected unto A549 cells, whereas MEb1+ was the most cytotoxic one when administered on MCF-7 cells. The viability percentages of HCT-116 cells were the lowest when treated with GEM1 while the least viability percentages of HFS cells were observed when MEa1 was subjected. The viability percentages of A549, MCF-7, HCT-116 and HFS cells were the highest when incubated with MEc1, GEM1, MEc1+ and MEc1, respectively.

Table 4.3 Cytotoxic screening of 1 µM of blank microemulsions (MEa1, MEb1 and MEc1), gemcitabine loaded-microemulsions (MEa1+, MEb1+ and MEc1+) and gemcitabine solution (GEM1) subjected unto non-small lung cancer cells (A549), breast cancer cells (MCF-7), colon cancer cells (HCT-116), and human foreskin cells (HFS) using sulphorhodamine B assay. The percentages of cell viabilities were expressed as $\bar{X}$ ± SD.

Formulations	% Cell Viability			
	A549	MCF-7	HCT-116	HFS
MEa1	52.70±5.31	45.95±4.51	54.76±2.15	50.42±8.12
MEa1+	68.88±5.14	34.92±4.46	65.64±7.52	80.26±5.42
MEb1	45.32±1.60	35.84±4.68	53.35±2.66	58.24±5.41
MEb1+	57.61±6.02	30.27±7.41	62.93±5.95	56.80±0.21
MEc1	76.68±8.41	46.45±10.39	63.00±7.55	83.63±4.17
MEc1+	64.03±6.40	37.80±4.79	87.33±6.92	62.94±3.26
GEM1	61.69±8.65	54.28±3.17	51.22±2.89	64.02±1.27

The responses of the selected cell lines were significantly different when treated with 1 μM of different microemulsion formulations and GEM1 as confirmed by the statistical analyses using two-factor ANOVA and identifying the *p*-values for pairwise *t*-tests (Table 4.4). The assessment revealed very highly significant differences in the viability percentages between MCF-7 cells and all of A549, HCT-116 and HFS cells, whereas the percentages of cell viability of A549 were significantly different from HFS. On the other hand, there were no significant differences in the viability percentages between HCT-116 cells and both of A549 and HFS cells. Among the drug formulations, there were very highly significant differences in the cytotoxic effect on A549, MCF-7, HCT-116 and HFS cells. The percentages of cell viability, when GEM1 subjected onto all of the cell lines, were very highly significantly different from all of the blank microemulsion formulas (MEa1, MEb1 and MEc1) and significantly different from drug loaded-microemulsions (MEb1+ and MEc1+). The only microemulsion formula that showed similar percentages of cell viability to GEM1 was MEa1+. The overall cytotoxicity of MEb1, MEb1+ and MEa1 were equivalent but were very highly significantly different from MEa1+, MEc1 and MEc1+. It is worth noting that MEc1 and MEc1+ did not have significant difference in cytotoxicity, when subjected onto the various cell lines.

Table 4.4 The statistical assessment of the differences in the percentages of cell viability between the A549 non-small lung cancer cells, MCF-7 breast cancer cells, HCT-116 colon cancer cells, and HFS human foreskin cells when treated with 1 µM of blank microemulsions (MEa1, MEb1 and MEc1), gemcitabine loaded-microemulsions (MEa1+, MEb1+ and MEc1+) and gemcitabine solution (GEM1) using two-factor ANOVA test and identifying the *p*-values for pairwise *t*-test.

***p*-values for pairwise *t*-tests of factor1**						
Factor 1	MEb1	MEa1	MEb1+	GEM1	MEa1+	MEc1+
MEa1	0.2391					
MEb1+	0.1158	0.6851				
GEM1	0.0001	0.0047	0.0140			
MEa1+	9.65×110^{-8}	7.66×10^{-6}	3.21×10^{-5}	0.0518		
MEc1+	3.68×10^{-8}	3.05×10^{-6}	1.31×10^{-5}	0.0288	0.7988	
MEc1	2.72×10^{-11}	2.49×10^{-9}	1.17×10^{-8}	.0001	0.0355	0.0627

***p*-values for pairwise *t*-tests of factor 2**				
Factor 2	MCF-7	A549	HCT 116	HFS
MCF-7				
A549	2.36×10^{-16}			
HCT 116	1.05×10^{-17}	0.3623		
HFS	9.04×10^{-20}	0.0203	0.1471	

[a] Factor 1 is the selected drug formula

[b] Factor 2 is the selected cell line

Note: Cells colored with orange have p-values with very highly significant differences between the groups while, green have significant differences among them.

In order to examine the effect of the concentration of drug formulations on the percentages of cell viability of A549, MCF-7, HCT-116 and HFS, 10 μM of blank microemulsions MEa10, MEb10 and MEc10, gemcitabine loaded-microemulsions MEa10+, MEb10+ and MEc10+ and GEM10 were applied. As shown in Table 4.5, the most cytotoxic formula administered onto A549 and HFS cells was MEb10+, while MEa10 and MEb10 were the most cytotoxic when subjected onto MCF-7 and HCT-116 cells, respectively. The least cytotoxic effect was observed when GEM10 was subjected onto A549, MCF-7, HCT-116 and HFS cells.

The responses of the selected cell lines to the drug formulations were very highly significantly different as statistically assessed using two-factor ANOVA test and identifying the *p*-values for pairwise *t*-tests (Table 4.6). Among the drug formulations, there were very highly significant differences between the cytotoxicity of GEM10 and all of the microemulsion formulations. Regarding the microemulsion formulations, there were no significant differences between the blank microemulsions, MEb10 and MEc10, and their drug loaded formulas, MEb10+ and MEc10+, when applied onto A549, MCF-7, HCT-116 and HFS cells. However, there were significant differences in the percentages of cell viability of all of the selected cell lines when treated with MEa10 and MEa10+. Regarding the blank microemulsions, the cytotoxicities were similar between MEb10 and both of MEa10 and MEc10 but were significantly different between MEa10 and MEc10. On the other hand, the percentages of cell viability of the various cell lines, when incubated with the drug load-microemulsions, MEb10+, were significantly different from both of MEa10+and MEc10+ , in spite of their equivalent toxicity.

Table 4.2 Cytotoxic screening of 10 µM of different microemulsion formulations subjected unto non-small lung cancer cells (A549), breast cancer cells (MCF-7), colon cancer cells (HCT-116), and human foreskin cells (HFS) using sulphorhodamine B assay. The The percentages of cell viabilities were expressed as $\bar{X}$ ± SD.

Formulations	% Cell Viability			
	A549	MCF-7	HCT-116	HFS
MEa10	13.07 ± 1.44	21.67±1.77	32.17±11.81	53.55±5.53
MEa10+	14.33±1.55	31.76±3.28	37.03±3.64	56.08±2.29
MEb10	13.31±0.78	26.18±1.76	31.29±7.80	56.44±3.71
MEb10+	11.15±.54	26.08±3.59	32.85±5.89	51.26±4.69
MEc10	15.11±2.12	29.22±1.19	36.93±4.74	54.03±2.54
MEc10+	16.07±0.58	34.71±7.71	37.12±3.37	56.56±5.24
GEM10	58.09±3.07	42.3±5.70	50.84±1.31	62.70±1.27

Table 4.3 The statistical assessment of the differences in the percentages of cell viability between the A549 non-small lung cancer cells, MCF-7 breast cancer cells, HCT-116 colon cancer cells and HFS human foreskin cells when treated with 10 µM of blank microemulsions (MEa10, MEb10 and MEc10), gemcitabine loaded-microemulsions (MEa10+, MEb10+ and MEc10+) and gemcitabine solution (GEM10) using two-Factor ANOVA test and identifying the *p*-values for pairwise *t*-test.

***p*-values for pairwise *t*-tests of factor 1**,						
Factor 1	MEa10	MEb10+	MEb10	MEc10	MEa10+	MEc10+
MEb10+	0.9014					
MEb10	0.3470	0.4134				
MEc10	0.0421	0.0555	0.2627			
MEa10+	0.0110	0.0152	0.0985	0.5854		
MEc10+	0.0014	.0020	0.0189	0.2037	0.4639	
GEM10	5.60×10^{-20}	8.27×10^{-20}	1.15×10^{-18}	4.89×10^{-17}	3.20×10^{-16}	4.23×10^{-15}

***p*-values for pairwise *t*-tests of factor 2**				
Factor 2	A549	MCF-7	HCT 116	HFS
A549				
MCF-7	3.63×10^{-11}			
HCT-116	1.03×10^{-17}	0.0001		
HFS	2.52×10^{-33}	3.08×10^{-25}	5.51×10^{-20}	

[a] Factor 1 is the selected drug formula

[b] Factor 2 is the selected cell line

Note: Cells colored with orange have p-values with very highly significant differences between the groups while, green have significant differences among them.

The comparisons between 1 and 10 μM of the entire drug formulations, subjected onto A549, MCF-7, HCT-116 and HFS cells, were demonstrated in Figures 4.2, 4.3, 4.4 and 4.5, respectively. The differences between the formulas administered onto each cell line were evaluated statistically using one-factor ANOVA test and measuring *p*-values for pairwise *t*-tests as illustrated in Tables 4.7, 4.8, 4.9, and 4.10. It was so obvious that there were no significant differences between GEM1 and GEM10 subjected onto all of the cell lines. In contrast, the microemulsion formulations showed discrepancies by changing concentrations.

As shown in Figure 4.2 and demonstrated statistically in Table 4.7, the viability percentages of A549 cells have decreased significantly when the concentrations of all of the microemulsion formulations increased from 1 to 10 μM. It is worth noting here that there were no significant differences in cytotoxicity effect between each blank microemulsion and its drug loaded-formula at 10 μM. However, there were highly significant differences between them at 1 μM. The cytotoxicity of MEa1+ and MEb1+ were highly significantly less than their blank formula, MEa and MEb, respectively. On the contrary, the cytotoxicity of MEc1+ was more than its blank formula, MEc1. It should be demonstrated that MEb10 and MEb10+ exhibited the most anti-proliferative activity against A549 cells.

Compared to A549 cells, the MCF-7 breast cancer cells responded differently to the microemulsion formulations as shown in Figure 4.3 and illustrated statistically in Table 4.8. The increase in the concentration of MEa+, MEb+ and MEc+ from 1 to 10 μM did not significantly affect the percentages of cell viability. Furthermore, the percentages of cell viability did not exhibit any differences between the blank microemulsion formulas (MEb1, MEb10, MEc1 and MEc10) and their gemicitabine loaded-formulas (MEb1+, MEb10+, MEc1+ and MEc10+).

Similar to A549 cells, the viability percentages of HCT-116 cells decreased significantly when all of the microemulsion formulations concentration increased from 1 to 10 μM as exemplified in Figure 4.4 and demonstrated statistically in Table 4.9. The cytotoxicity of MEa1 and MEc1 were significantly more than their drug loaded formulas, MEa1+ and MEc1+, while the cytotoxicity of MEb1 and MEb1+ were equivalent. There were no significant differences between the blank microemulsions (MEa10, MEb10 and MEc10) and their drug- loaded formulas (MEa10+, MEb10+ and MEc10+).

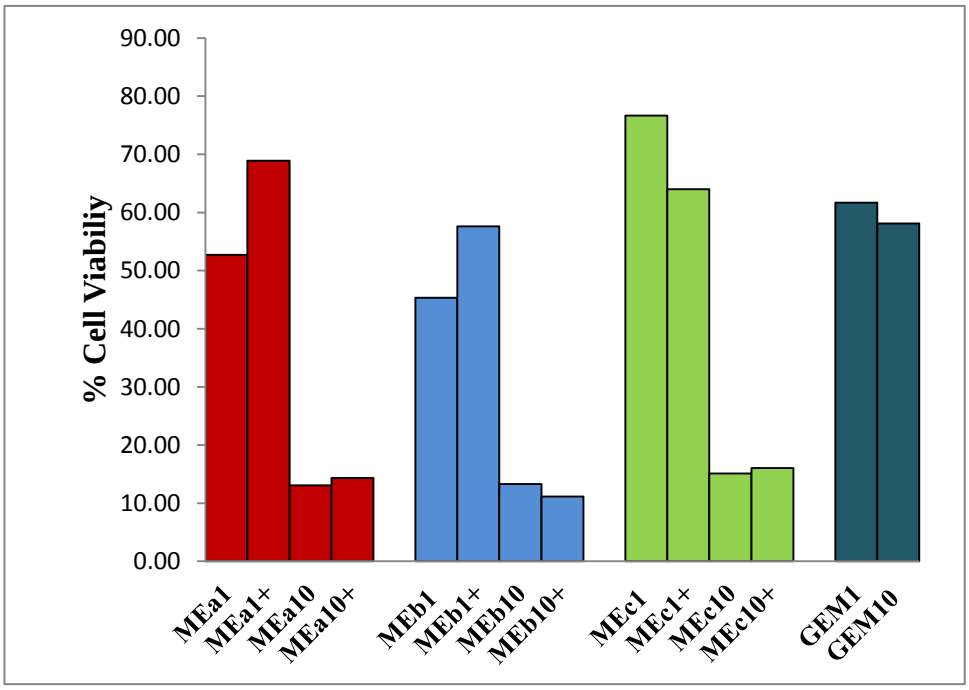


Figure 4.2 Percentages of cell viability of 1 and 10 µM of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM), subjected onto non-small cell lung cancer (A549). It should be noted that all of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively.

Table 4.4 Statistical evaluation of the differences in the cytotoxicity effect between 1 and 10 µM of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM) applied onto A549 non-small cell lung cancer cells using one-factor ANOVA and identifying *p*-values using pairwise *t*-tests.

	MEb10+	MEa10	MEb10	MEa10+	MEc10	MEc10+	MEb1	MEa1	MEb1+	GEM10	GEM1	MEc1+
MEb10+												
MEa10	0.6155											
MEb10	0.5723	0.9498										
MEa10+	0.4073	0.7414	0.7893									
MEc10	0.3038	0.5937	0.6376	0.8380								
MEc10+	0.2037	0.4341	0.4714	0.6488	0.8014							
MEb1	8.35E-10	2.78E-09	3.24E-09	6.22E-09	1.03E-08	1.94E-08						
MEa1	1.13E-11	3.30E-11	3.78E-11	6.79E-11	1.07E-10	1.88E-10	0.0610					
MEb1+	8.27E-13	2.24E-12	2.54E-12	4.37E-12	6.66E-12	1.13E-11	0.0030	0.2037				
GEM10	6.48E-13	1.74E-12	1.97E-12	3.38E-12	5.14E-12	8.66E-12	0.0021	0.1642	0.8999			
GEM1	1.09E-13	2.79E-13	3.14E-13	5.24E-13	7.78E-13	1.27E-12	0.0002	0.0243	0.2896	0.3490		
MEc1+	3.61E-14	8.94E-14	1.00E-13	1.64E-13	2.41E-13	3.88E-13	3.16E-05	0.0056	0.1005	0.1273	0.5409	
MEa1+	4.07E-15	9.47E-15	1.05E-14	1.67E-14	2.38E-14	3.72E-14	9.66E-07	0.0002	0.0058	0.0080	0.0671	0.2091
MEc1	1.63E-16	3.49E-16	3.84E-16	5.81E-16	8.00E-16	1.19E-15	4.94E-09	7.19E-07	2.43E-05	3.44E-5	0.0005	0.0023

Note: All of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively. Cells colored with pink have p-values with very highly significant differences between the groups while violet cells have highly significant differences among them.

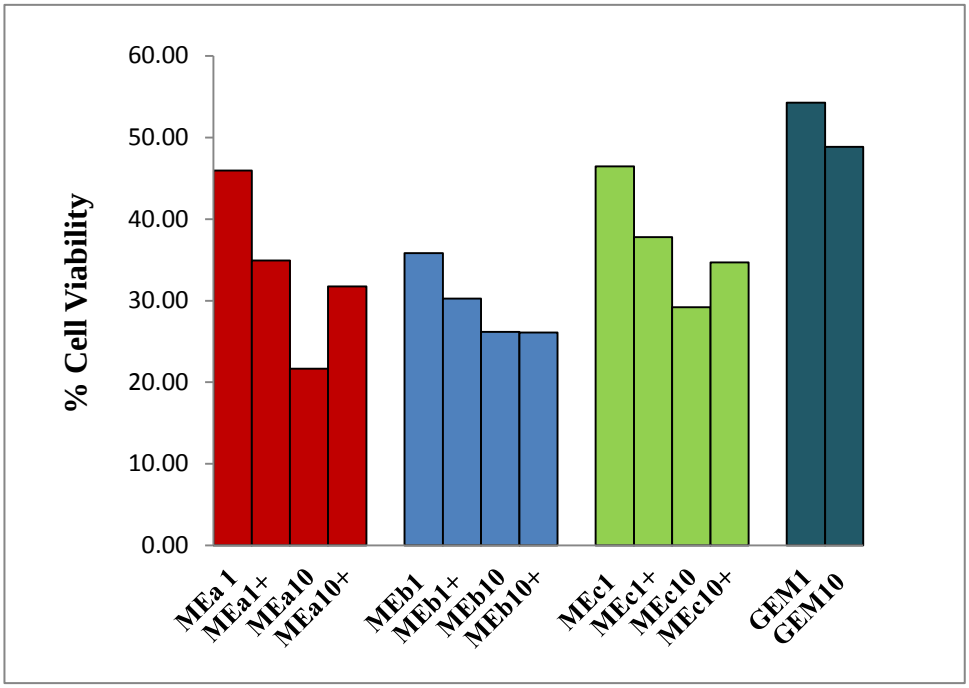


Figure 4.3 Percentages of cell viability of different formulations of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM) on breast cancer cells (MCF-7). It should be noted that all of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively.

Table 4.5 Statistical evaluation of the differences in the cytotoxicity effect between 1 and 10 µM of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM) applied onto MCF-7 human breast cancer cells using one-factor ANOVA and identifying *p*-values using pairwise *t*-tests.

	MEa10	MEb10+	MEb10	MEc10	MEa10+	MEb1+	MEc10+	MEa1+	MEb1	MEc1+	MEa1	MEc1	GEM10
MEa10													
MEb10+	0.3094												
MEb10	0.2990	0.9818											
MEc10	0.0874	0.4677	0.4816										
MEb1+	0.0531	0.3334	0.3446	0.8059									
MEa10+	0.0249	0.1928	0.2004	0.5545		0.7289							
MEc10+	0.0048	0.0525	0.0551	0.2082	0.4957	0.3071							
MEa1+	0.0043	0.0473	0.0496	0.1914	0.4650	0.2846	0.9601						
MEb1	0.0025	0.0297	0.0312	0.1311	0.3467	0.2018	0.7915	0.8303					
MEc1+	0.0007	0.0103	0.0109	0.0536	0.1675	0.0881	0.4734	0.5045	0.6492				
MEa1	4.14E-06	0.0001	0.0001	0.0005	0.0025	0.0010	0.0135	0.0152	0.0249	0.0663			
MEc1	3.01E-06	0.0001	0.0001	0.0004	0.0018	0.0007	0.0102	0.0115	0.0191	0.0521	0.9075		
GEM10	6.57E-07	1.07E-05	1.14E-05	0.0001	0.0004	0.0002	0.0025	0.0028	0.0049	0.0148	0.4989	0.5746	
GEM1	2.45E-08	3.52E-07	3.74E-07	2.51E-06	1.27E-05	4.91E-06	0.0001	0.0001	0.0002	0.0006	0.0605	0.0765	0.2141

Note: All of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively.Cells colored with pink have p-values with very highly significant differences between the groups , while violet cells have highly significant differences among them.

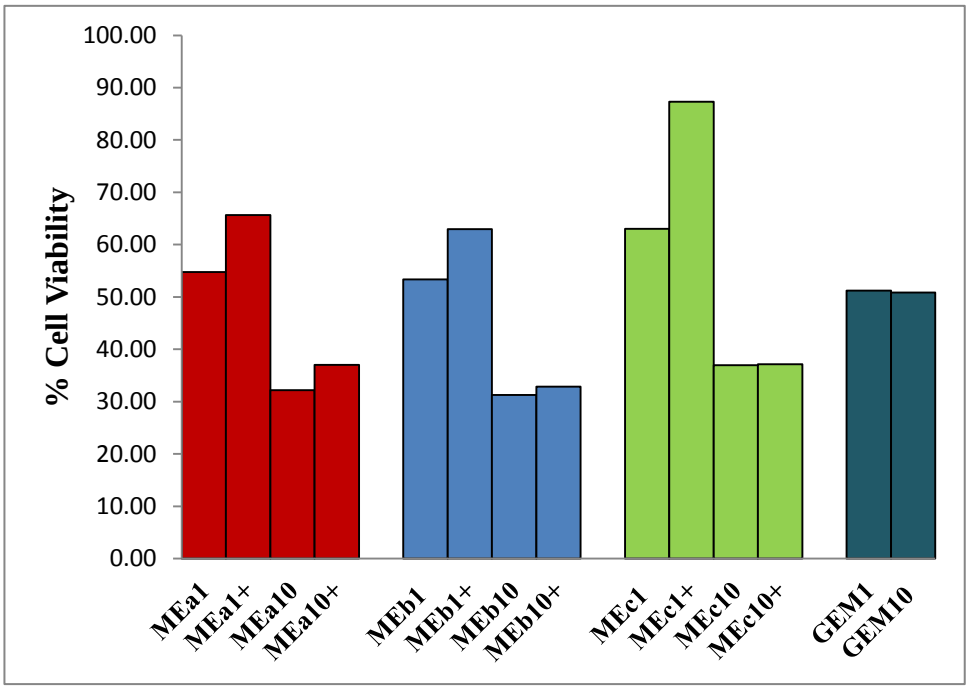


Figure 4.4 Percentages of cell viability of different formulations of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM) on colon cancer cells (HCT-116). It should be noted that all of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively.

Table 4.6 Statistical evaluation of the differences in the cytotoxicity effect between 1 and 10 µM of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM) applied onto HCT-116 colon cancer cells using one-factor ANOVA and identifying *p*-values using pairwise *t*-tests.

	MEb10	MEa10	MEb10+	MEc10	MEa10+	MEc10+	GEM10	GEM1	MEb1	MEa1	MEb1+	MEc1	MEa1+
MEb10													
MEa10	0.8591												
MEb10+	0.7525	0.8902											
MEc10	0.2581	0.3378	0.4103										
MEa10+	0.2502	0.3282	0.3994	0.9843									
MEc10+	0.2425	0.3188	0.3887	0.9685	0.9843								
GEM10	0.0004	0.0007	0.0010	0.0082	0.0086	0.0090							
GEM1	0.0003	0.0005	0.0008	0.0067	0.0071	0.0074	0.9375						
MEb1	0.0001	0.0002	0.0002	0.0023	0.0024	0.0025	0.6113	0.6670					
MEa1	4.71E-05	0.0001	0.0001	0.0011	0.0011	0.0012	0.4283	0.4746	0.7740				
MEb1+	5.08E-07	8.18E-07	1.19E-06	1.14E-05	1.20E-05	1.27E-05	0.0195	0.0234	0.0596	0.1053			
MEc1	4.91E-07	7.90E-07	1.15E-06	1.10E-05	1.16E-05	1.23E-05	0.0190	0.0227	0.0580	0.1028			
MEa1+	1.19E-07	1.90E-07	2.74E-07	2.54E-06	2.68E-06	2.82E-06	0.0052	0.0063	0.0178	0.0342			
MEc1+	4.25E-12	6.13E-12	8.16E-12	4.78E-11	4.99E-11	5.21E-11	3.88E-08	4.74E-08	1.45E-07	3.09E-07	2.82E-05	2.92E-05	0.0001

Note: All of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively. Cells colored with pink have p-values with very highly significant differences between the groups while, violet cells have highly significant differences among them.

The cytotoxicity of the drug formulations were scanned against HFS normal human foreskin cells with the aim to find out the safest formula that has minimized side effects. According to Figure 4.5 and the statistical analyses demonstrated in Table 4.10. MEa1+ and MEc1 were having the least cytotoxic effect with very high significant differences with the rest of the drug formulations used in this study. Furthermore, the cytotoxicity of MEa1 was significantly more than that of GEM1, GEM10 and MEc1+. The cytotoxicity of GEM1 was significantly less than all of the microemulsion formulations used at 10 µM, while GEM10 cytotoxicity was significantly less than MEa10, MEc10 and MEb10+. There were no significant differences between the cytotoxicity of the blank microemulsions (MEa10, MEb1, MEb10 and MEc10) and their drug loaded formula (MEa10+, MEb1+, MEb10+ and MEc10+).

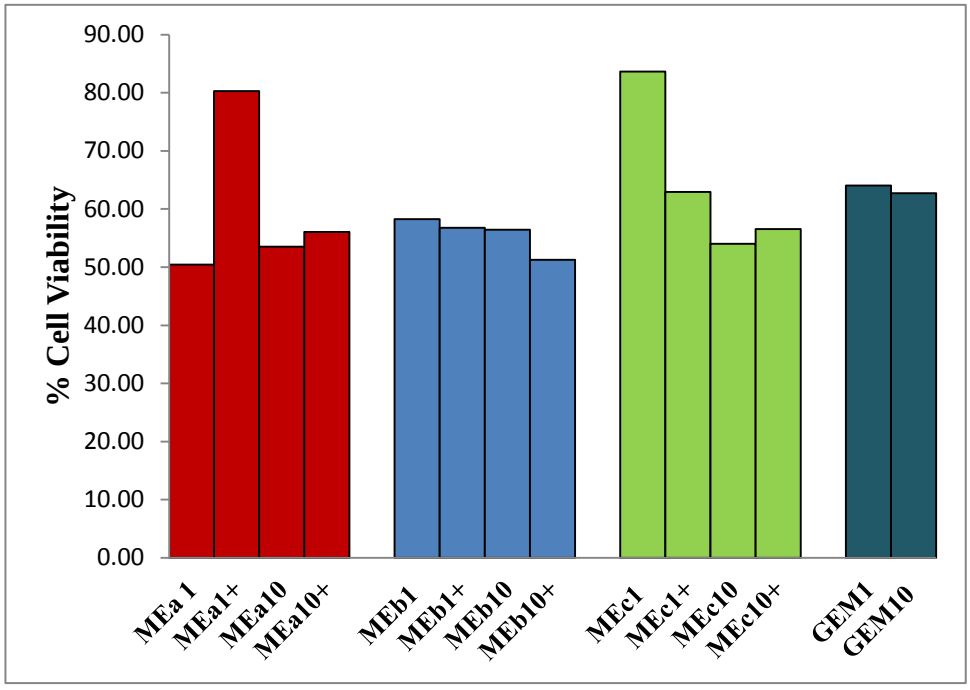


Figure 4.5 Percentages of cell viability of different formulations of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM) on human foreskin cells (HFS). It should be noted that all of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively.

Table 4.7 Statistical evaluation of the differences in the cytotoxicity effect between 1 and 10 µM of blank microemulsions (MEa, MEb and MEc), gemcitabine-loaded microemulsions (MEa+, MEb+ and MEc+) and gemcitabine solution (GEM) applied onto HFS human foreskin cells using one-factor ANOVA and identifying *p*-values using pairwise *t*-tests.

	MEa1	MEb10+	MEa10	MEc10	MEa10+	MEb10	MEc10+	MEb1+	MEb1	GEM10	MEc1+	GEM1	MEa1+
MEa1													
MEb10+	0.8130												
MEa10	0.3826	0.5221											
MEc10	0.3148	0.4392	0.8924										
MEa10+	0.1200	0.1832	0.4796	0.5665									
MEb10	0.0991	0.1535	0.4198	0.5006	0.9192								
MEc10+	0.0928	0.1445	0.4009	0.4796	0.8924	0.9730							
MEb1+	0.0813	0.1278	0.3648	0.4392	0.8393	0.9192	0.9461						
MEb1	0.0349	0.0577	0.1940	0.2424	0.5441	0.6128	0.6366	0.6853					
GEM10	0.0017	0.0031	0.0150	0.0205	0.0710	0.0869	0.0928	0.1057	0.2172				
MEc1+	0.0014	0.0026	0.0127	0.0175	0.0619	0.0760	0.0813	0.0928	0.1940	0.9461			
GEM1	0.0006	0.0012	0.0061	0.0085	0.0324	0.0404	0.0434	0.0501	0.1127	0.7103	0.7611		
MEa1+	3.35E-09	5.98E-09	2.99E-08	4.22E-08	1.88E-07	2.46E-07	2.69E-07	3.22E-07	9.52E-07	2.92E-05	3.52E-05	0.0001	
MEc1	3.56E-10	6.17E-10	2.84E-09	3.95E-09	1.64E-08	2.12E-08	2.31E-08	2.74E-08	7.78E-08	2.17E-06	2.61E-06	5.99E-06	0.3476

Note: All of the formulations were designated by 1 and 10 when used at 1 and 10 µM, respectively. Cells colored with pink have p-values with very highly significant differences between the groups, while violet cells have highly significant differences among them.

4.3 Characterization of cell morphology using light microscope

Morphology is one important indicator of the status of cells. In order to understand the mechanism of cell death of the blank and drug-loaded microemulsion formulations at a concentration of 1 and 10 µM when administered into A549, MCF-7, HCT-116 and HFS cells for 48 h, the cells morphologies were analyzed using light microscope. In general, slight changes in cells shape, decreased total number of cells and increased intracellular space of the cells treated with 1 µM of blank and loaded-microemulsion formulations were observed compared with the untreated cells whereas cells treated with 10 µM have shown dramatic changes in shape, extremely increased intracellular space and clearance of cells (Figures 4.6 - 4.13). On the other hand, there was no noticeable difference between the cells treated with either 1 or 10 µM of gemcitabine solution except little changes in the morphology of the cells.

Both A549 and MCF-7 cells were not obviously affected by all of the formulations at 1 µM as shown in Figures 4.6 and 4.8. They only displayed earlier stages of apoptosis as more intracellular spaces were observed without noticeable changes in the shape of the cells. In contrast, they were more affected by the entire formulations at 10 µM, shown in Figures 4.7 and 4.9, as they exhibited late stages of apoptosis through the complete formation of fragmented apoptotic bodies resulted from the enormous killed cells.

On the other hand, HCT-116 cells treated with the blank and gemcitabine loaded-microemulsion formulations showed different levels of cell death as demonstrated in Figures 4.10 and 4.11. The treated cells with 10 µM of blank microemulsion formulas of both MEa10 and MEc10, and the entire formulations of

1 µM of blank and drug- loaded microemulsion showed earlier stages of apoptosis as they shrunk and their chromatin get more condensed. On the other hand, the 10 µM of blank microemulsion MEb10 had clearly killed most of the cells suggesting that the cells undergone late stage of apoptosis. Interestingly, the gemcitabine loaded-microemulsion MEb10+ formula showed also the maximum effect on the cells as more condensed chromatin and apoptotic bodies were observed compared with the other drug loaded-formulas MEa10+ and MEc10+ at which their cells undergone earlier stages of apoptosis. Noting that cells incubated with MEa10+ were having slight formation of apoptotic bodies while the cells treated with MEc10+ shrunk and their chromatin were more condensed.

In contrast, the HFS human foreskin cells were noticeably more affected by the treatment with the 1µM of blank and gemcitabine loaded- microemulsion formulas as shown in Figures 4.12 and 4.13. The cells were mostly affected by MEa1, MEb1 and MEb1+ as more intracellular spaces and decrease in the number of cells were seen, whereas cells treated with 10µM of blank and gemcitabine loaded-microemulsion formulations have slightly decreased in their total number and less intracellular spaces have displayed between them. It should be mentioned here that the blank microemulsion formulas have had less effect than the gemcitabine loaded-microemulsion. Nevertheless, there were no signs of apoptosis observed with either blank or drug loaded-microemulsion.

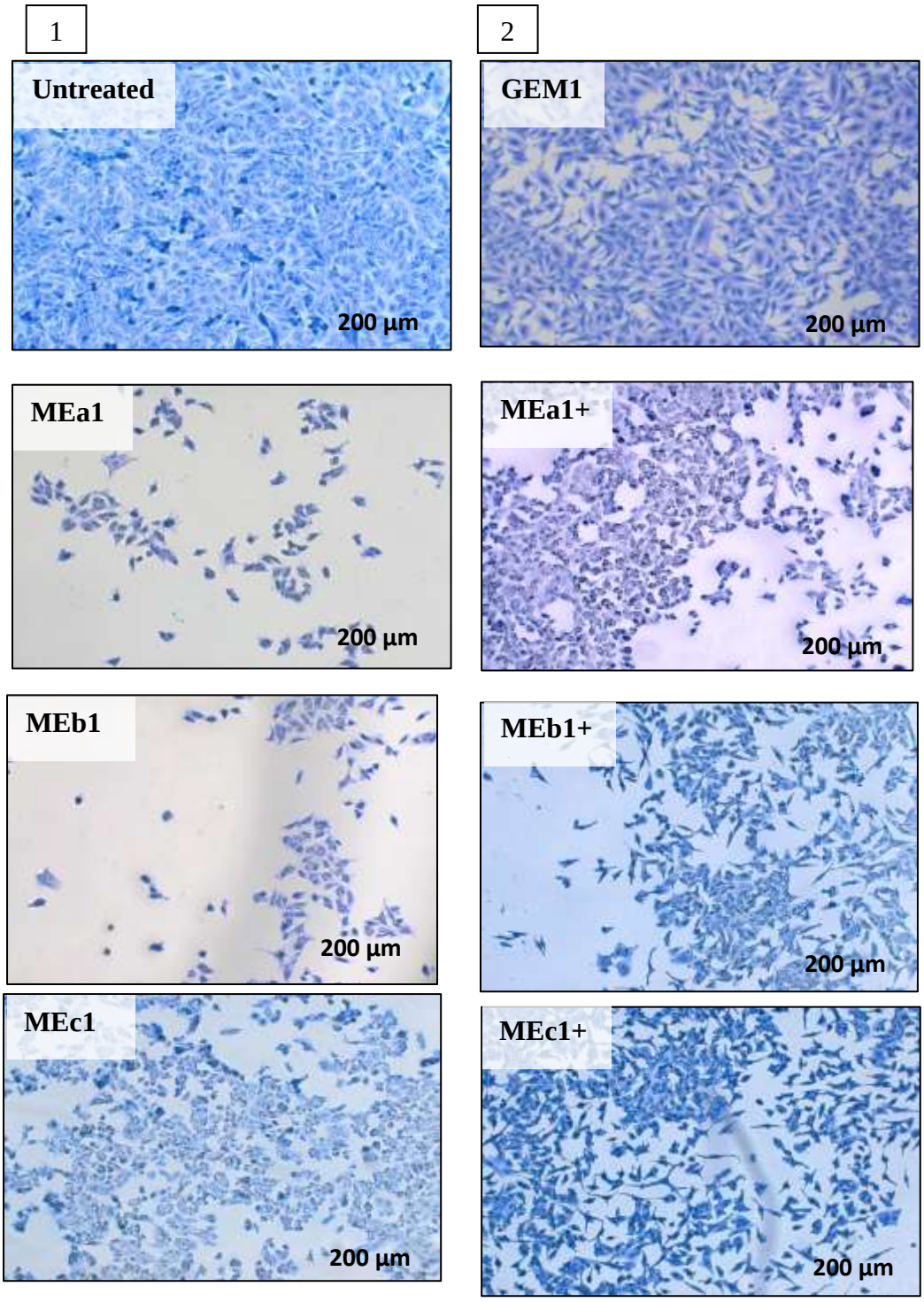


Figure 4.6 Light microscopy images showing the morphological changes in A549 non-small cell lung cancer treated with 1µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

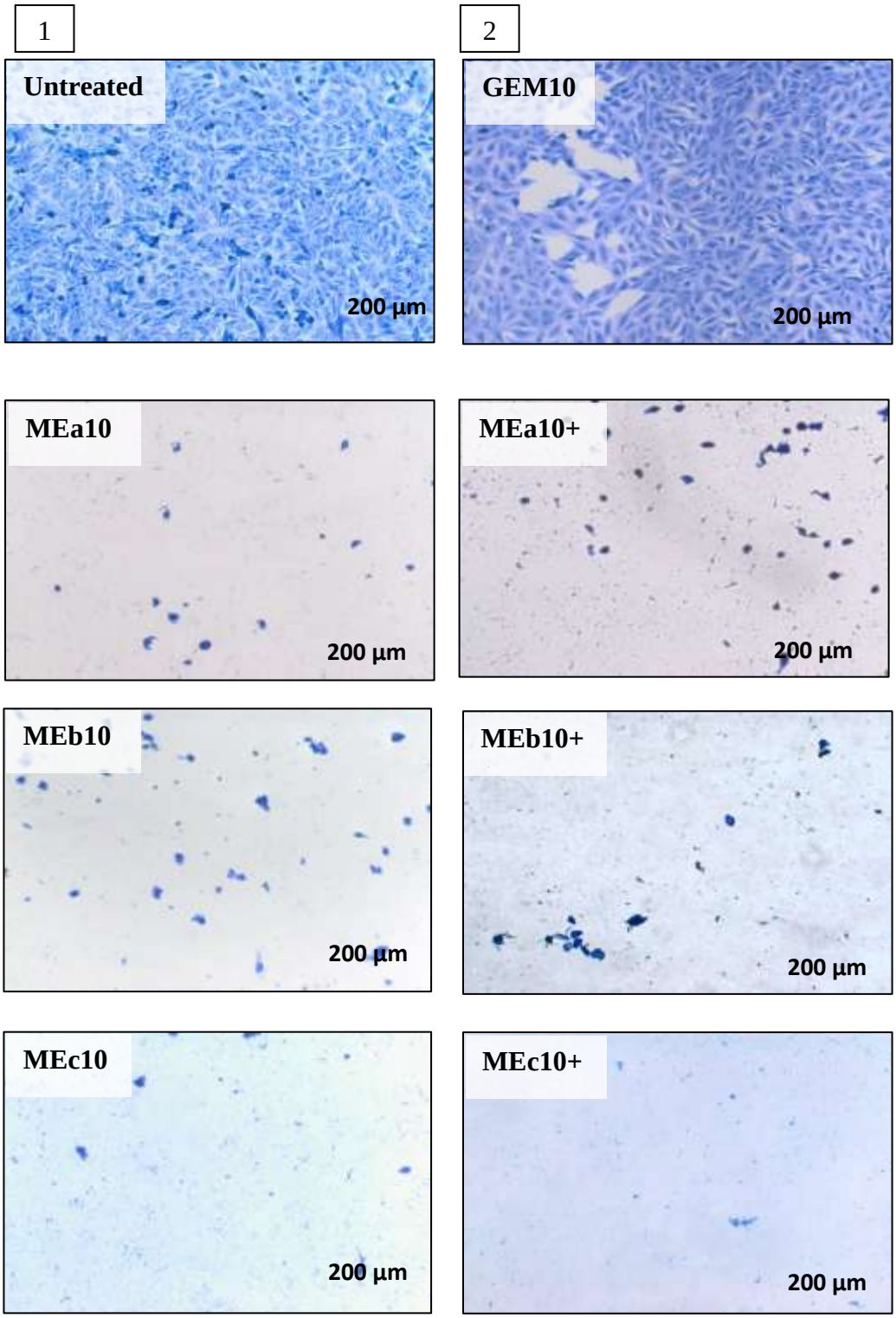


Figure 4.7 Light microscopy images showing the morphological changes in A549 non-small cell lung cancer treated with 10µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

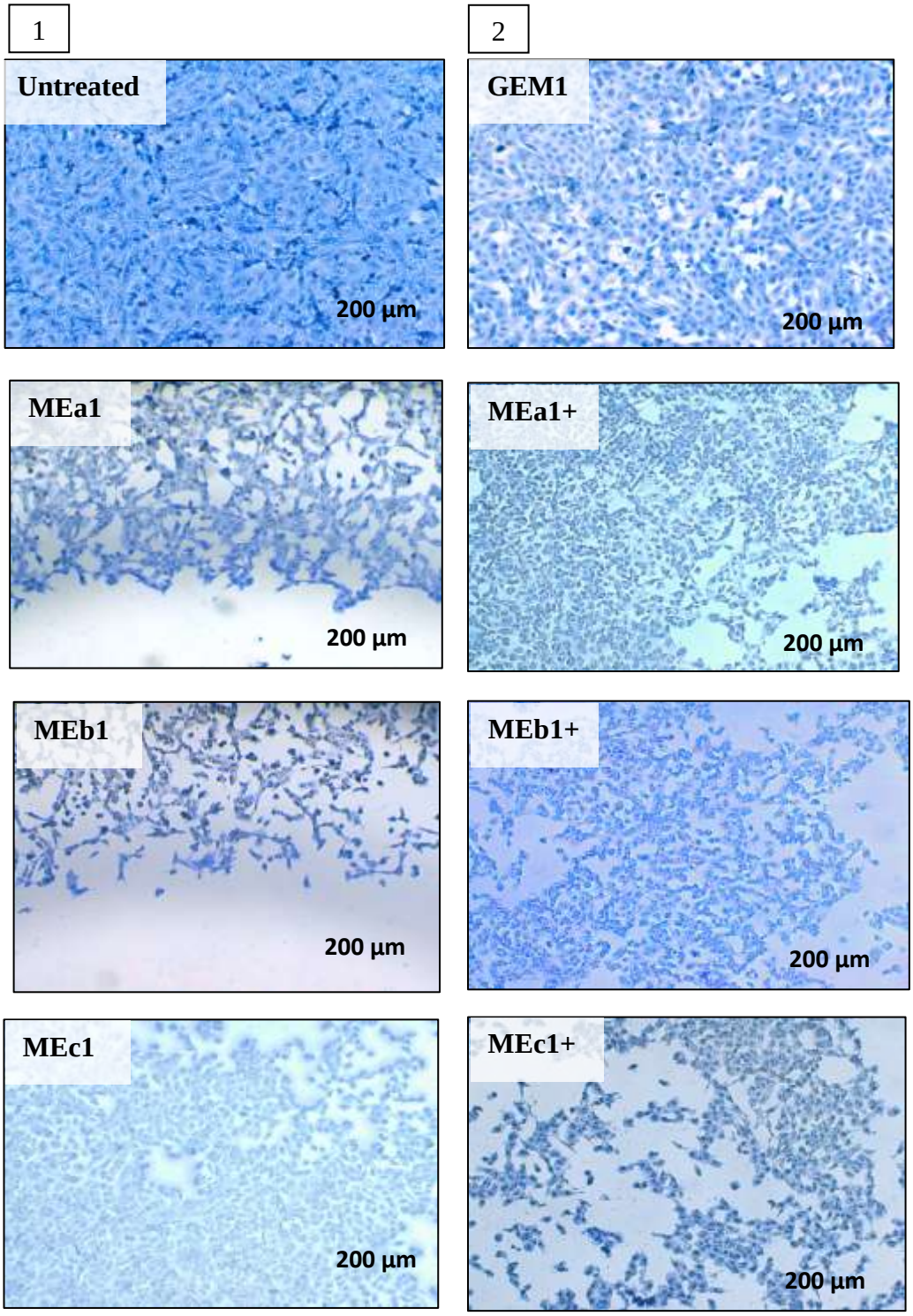


Figure 4.8 Light microscopy images showing the morphological changes in MCF-7 human breast cancer cells treated with 1µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

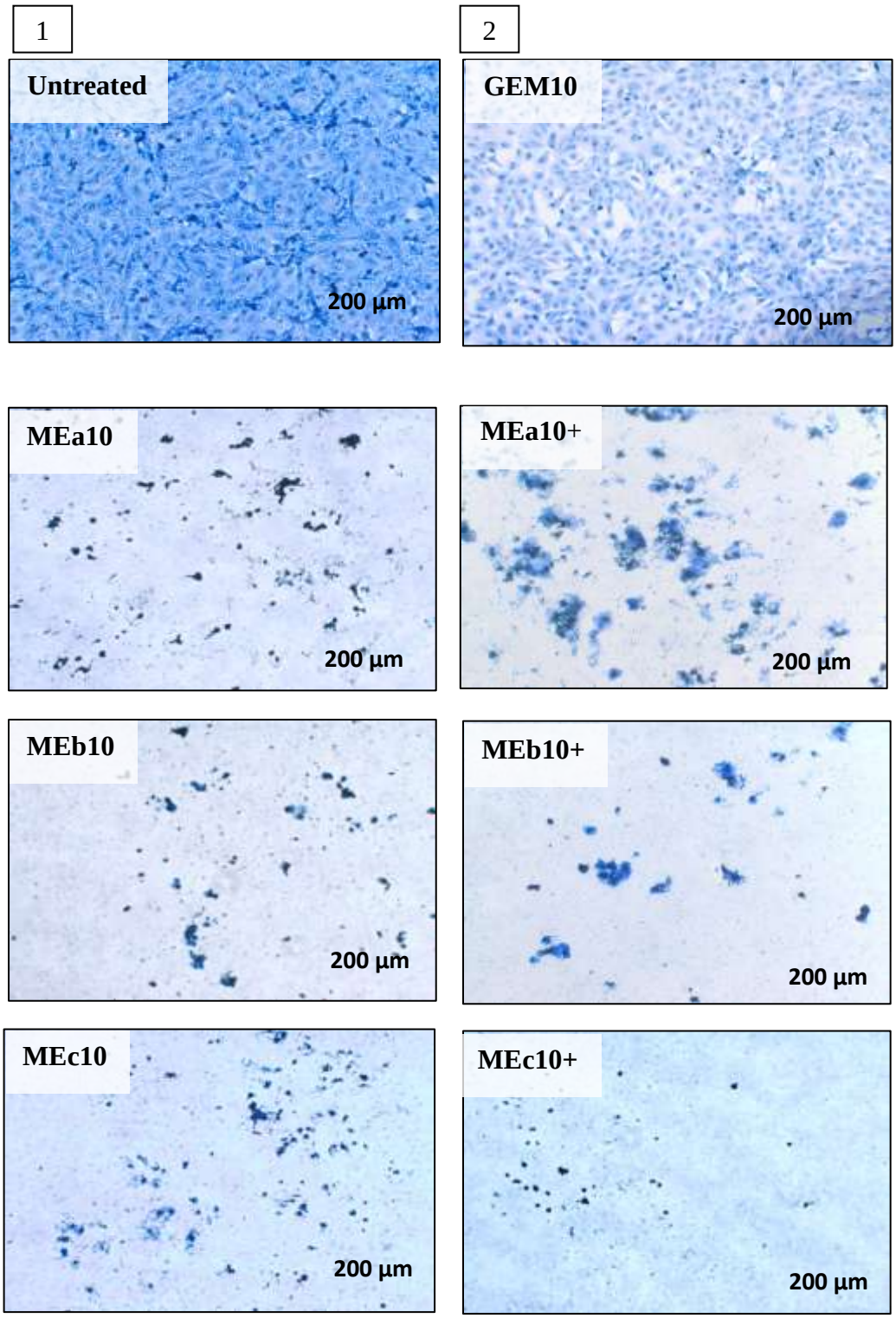


Figure 4.9 Light microscopy images showing the morphological changes in MCF-7 human breast cancer cells treated with 10µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

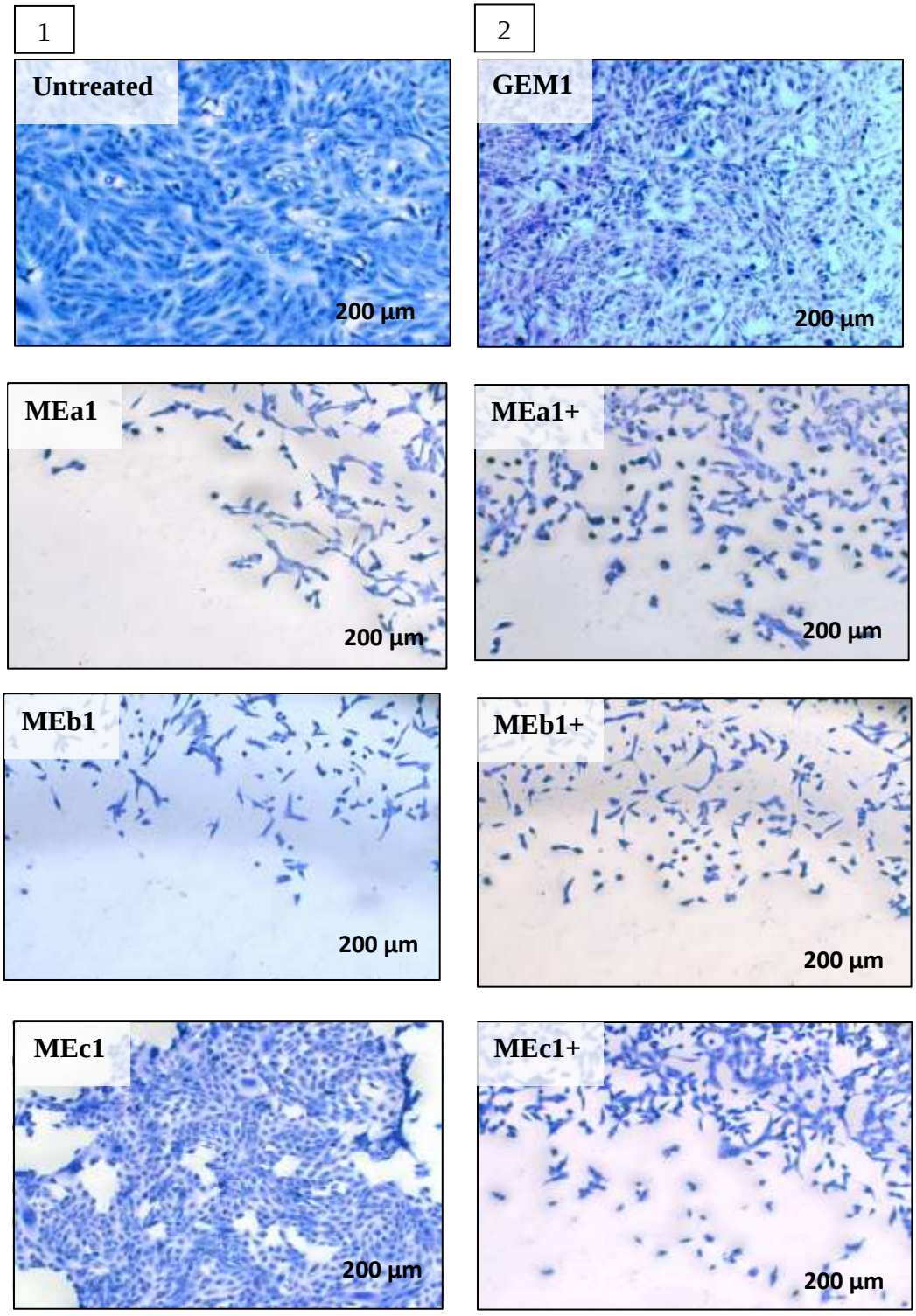


Figure 4.10 Light microscopy images showing the morphological changes in HCT-116 colon cancer cells treated with 1µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

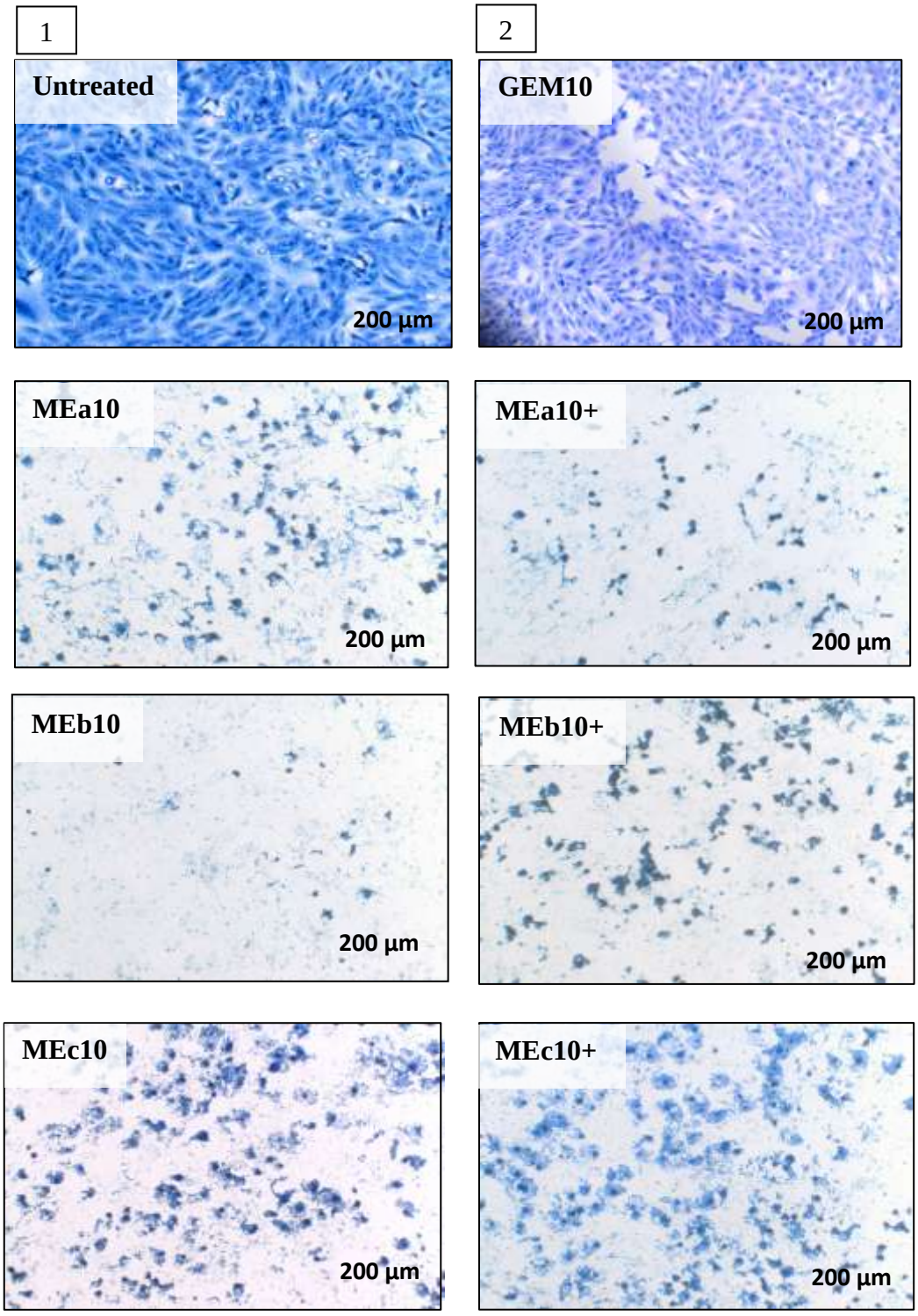


Figure 4. 2 Light microscopy images showing the morphological changes in HCT-116 colon cancer cells treated with 10µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

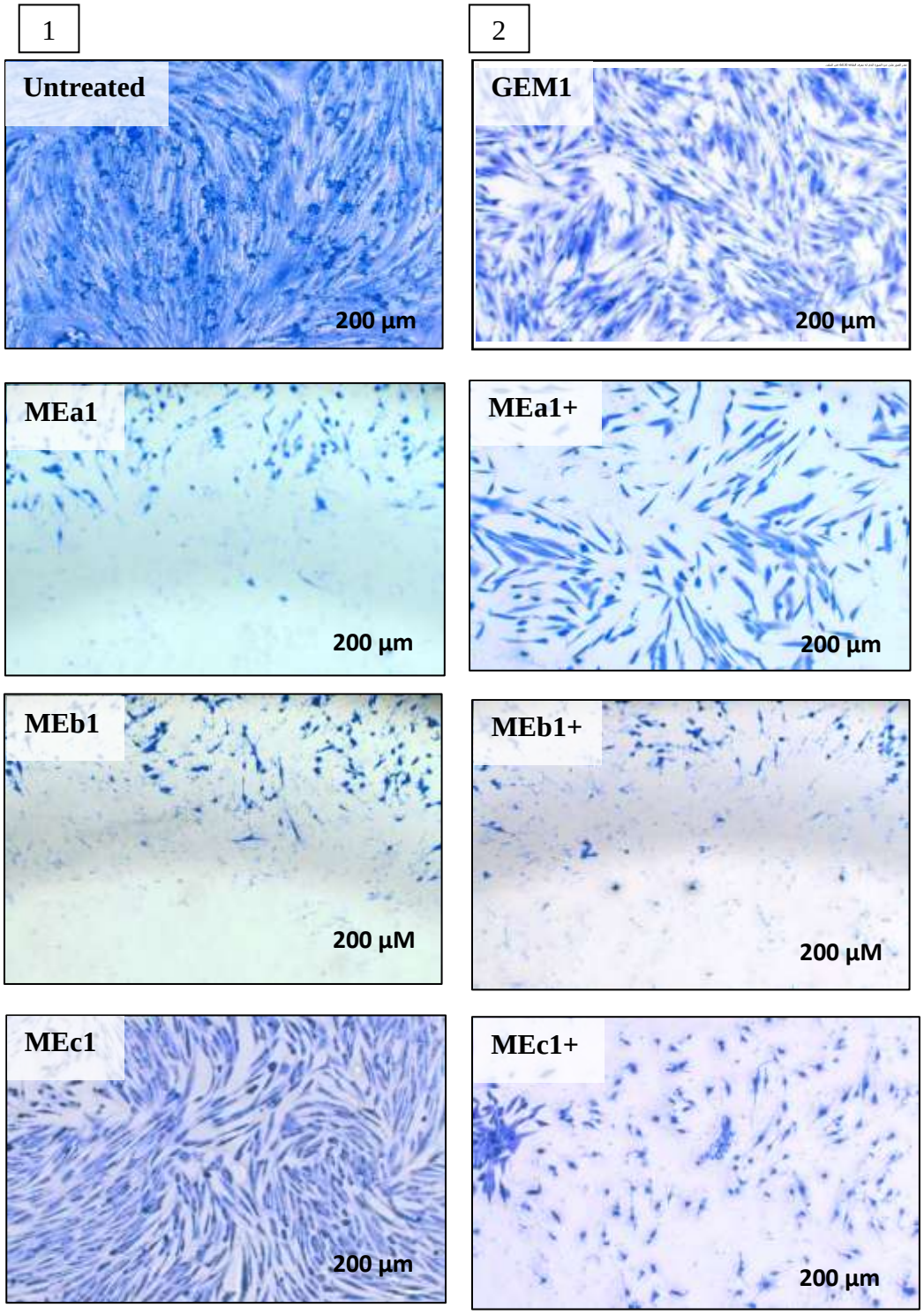


Figure 4.3 Light microscopy images showing the morphological changes in HFS human foreskin cells treated with 1µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

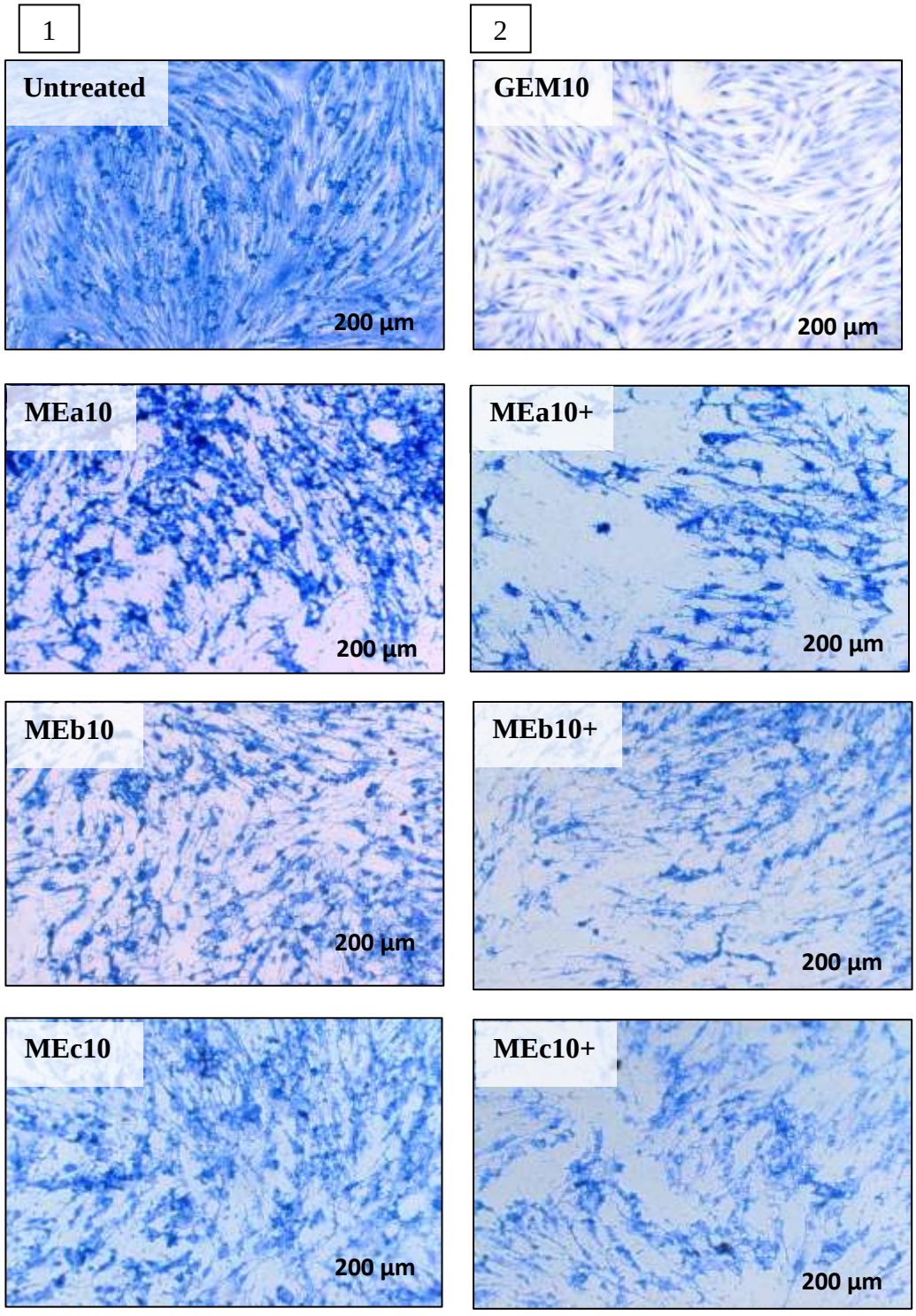


Figure 4.4 Light microscopy images showing the morphological changes in HFS human foreskin cells treated with 10µM formulations of (1) blank microemulsion and (2) gemcitabine – loaded microemulsion. Images were magnified at 200µm.

4.4 Effect of microemulsion formulations on apoptosis induction of cancer cells

After the treatment of A549, MCF-7, HCT-116 and HFS with 10μM of gemcitabine solution GEM10 and formula b of both blank microemulsion MEb10 and gemcitabine-loaded microemulsion MEb10+ for 48 h, the cells were labeled with annexin V conjugated with fluorescein isothiocyanate and PI propidium iodide, then viewed by fluorescence microscopy. As shown in Figures 4.14, 4.15 and 4.16, the untreated cells didn't stain positively with neither dyes which indicates the viability of cells, while all the treated cells with GEM10, MEb10 and MEb10+ were stained positively green fluorescent with annexin FITC but not with PI which implies signs of apoptosis with no detectable necrotic effect due to the externalization of phosphatidylserine (PS) caused by the cell surface outbreak. In contrast, it was found that treated HFS human foreskin cells had less apoptotic cells than the cancer cells as very few cells were lightly stained with green fluorescent.

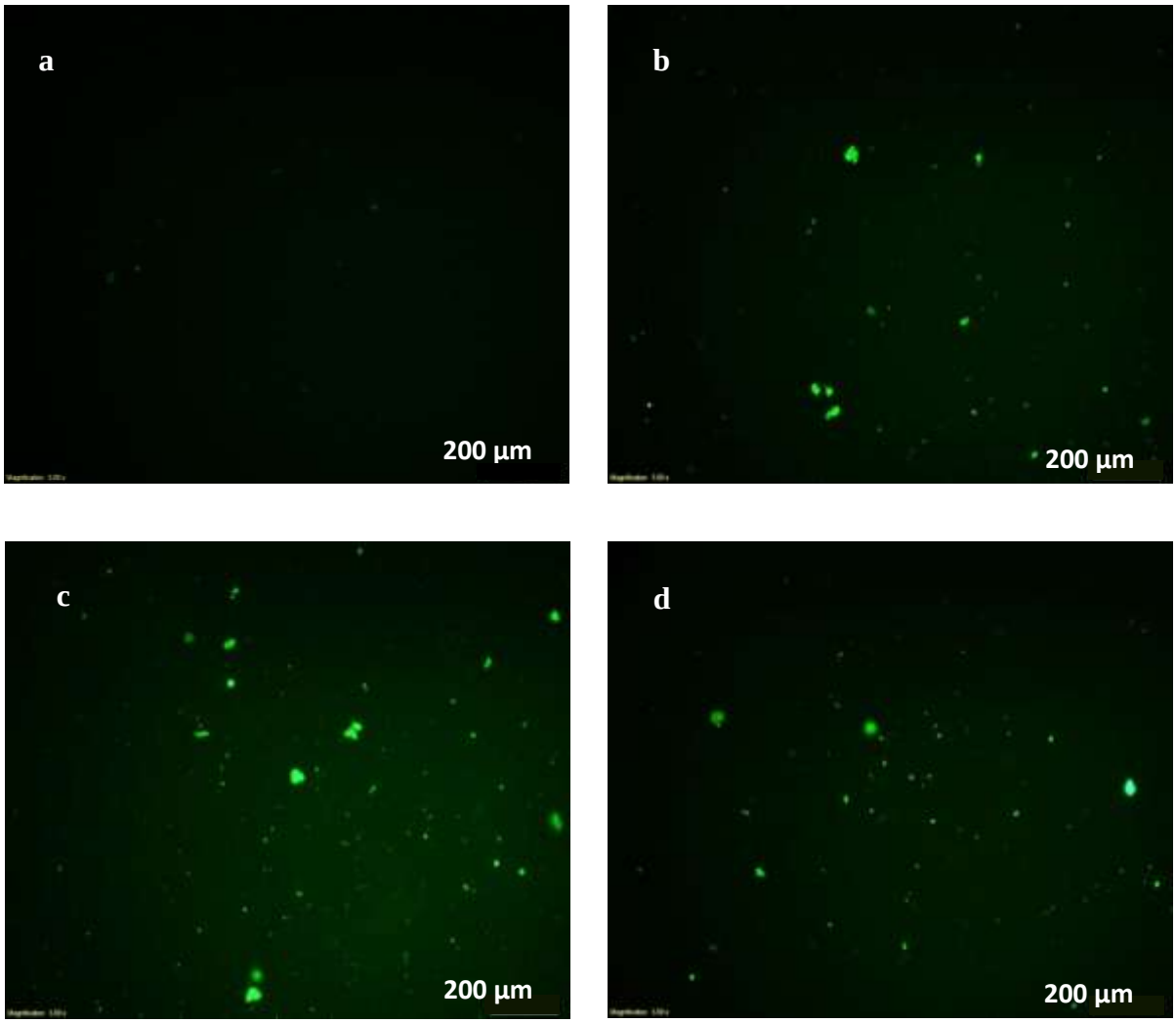


Figure 4.5 Fluorescent microscopic images of A549 non-small cell lung cancer cells labeled with Annexin-FITC and propidium iodide, magnified at 200 µm.(a) Untreated cells, cells treated with: (b) gemcitabine solution GEM10 , (c) formula b of blank microemulsion MEb10, and (d) formula b of gemcitabine- loaded microemulsion MEb10+.

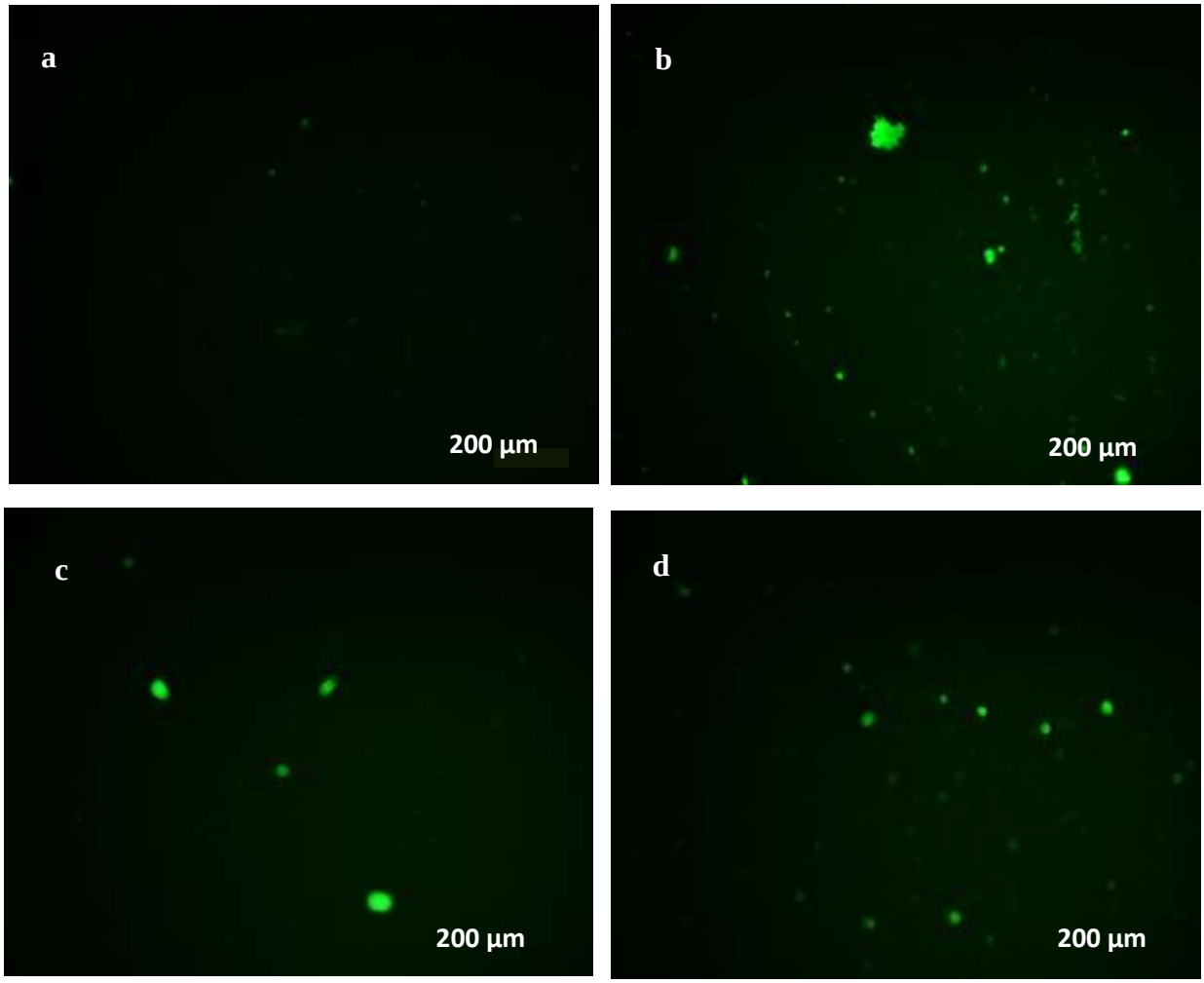


Figure 4.6 Fluorescent microscopic images of MCF-7 breast cancer cells labeled with Annexin-FITC and propidium iodide, magnified at 200 µm. . (a) Untreated cells, cells treated with: (b) gemcitabine solution GEM10, (c) formula b of blank microemulsion MEb10, and (d) formula b of gemcitabine-loaded microemulsion MEb10+.

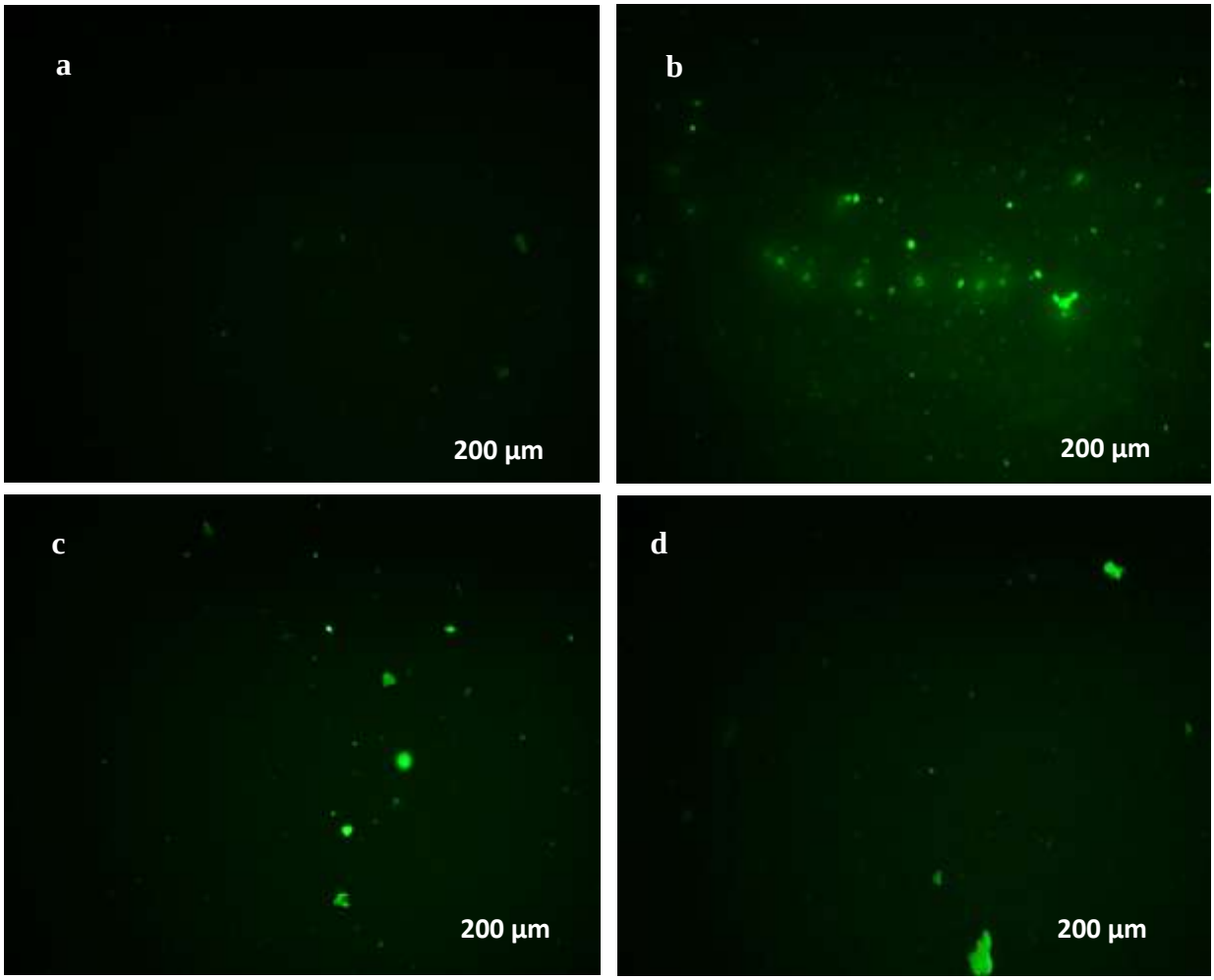


Figure 4.7 Fluorescent microscopic images of HCT-116 colon cancer cells labeled with Annexin-FITC and propidium iodide, magnified at 200 µm.. (a) Untreated cells, cells treated with: (b) gemcitabine solution GEM10, (c) formula b of blank microemulsion MEb10, (d) formula b of gemcitabine- loaded microemulsion MEb10+.

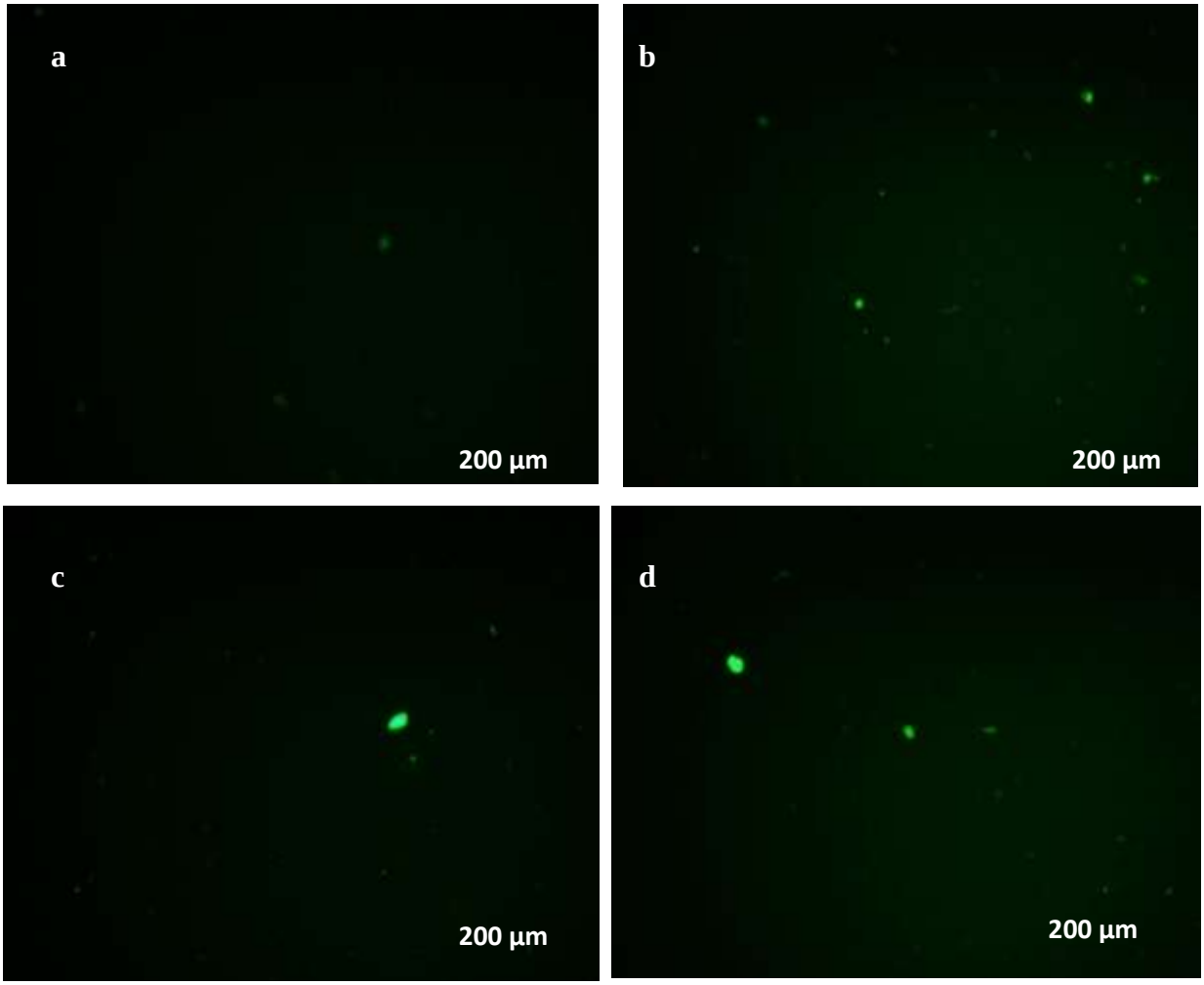


Figure 4.8 Fluorescent microscopic images HFS human foreskin cells labeled with Annexin-FITC and propidium iodide, magnified at 200 µm. (a) Untreated cells, cells treated with: (b) gemcitabine solution GEM10 , (c) formula b of blank microemulsion MEb10, and (d) formula b of gemcitabine- loaded microemulsion MEb10+.

4.5 Cell morphology and ultrastructure of A549 using Transmission Electron Microscope (TEM)

In order to elucidate the exact mechanism of cell death caused by the exposure of the A549 non-small cell lung cancer cells to 10 μM of gemcitabine solution (GEM10), blank microemulsion (MEb10) and gemcitabine loaded-microemulsion (MEb10+) for 48 h, TEM was utilized. Untreated cells have been considered as control as shown in Figure 4.18. Cells were rounded and contained few autolysosomes (AL) which are mature autophagosomes, membrane-bound compartment containing cytoplasmic material and/or organelles, fused with lysosomes, which are vesicles that contain digestive enzymes that break down waste and foreign materials.

Few cells were obtained when treated with GEM10, one of which was elongated and revealed early signs of apoptosis as observed in the whole cell in Figure 4.19. A section of the whole cell was further magnified, clearly seen in Figure 4.20 a, in order to show the presence of few AL's, lysosomes (L) and perixosomes (P) which are small, membrane enclosed organelles that contain enzymes involved in a variety of metabolic reactions, including several aspects of energy metabolism. Another section of the whole cell was enlarged, shown in Figure 4.20b, to demonstrate the formation of membrane blebbing and apoptotic bodies which are small sealed membrane vesicles that are produced from cells undergoing cell death by apoptosis.

Another obtained cell from GEM10 treatment has revealed late signs of apoptosis as displayed in Figure 4.21. Two sections of the whole cell were magnified to exhibit the chromatid fragments (CH) and the condensed mitochondria (M) (Figure 4.22a) and to illustrate the integration of the apoptosis with autophagy pathway (Figure 4.22b) as viewed through the formation of early autophagosomes (EAP), which are cytoplasmic constituents of cells engulfed within a cytoplasmic vacuole, small vesicles (sv), pre-autophagosomes (PAP), which are a punctuate structure localized in the vicinity of the vacuole that is required for the formation of autophagosomes, AL's that included swollen mitochondria and endoplasmic reticulum, and Whorl, which is a large degradative endosome, featured by a point of entry from an external, neighboring structure and multiple convoluted membranes which usually spans many sections. In the third GEM10 treated cell, displayed in Figure 4.23, late signs of apoptosis were seen through the extensive formation of membrane blebbing and apoptotic bodies, shrunken nucleolus (N) and pinocytic invagination of the nuclear membrane.

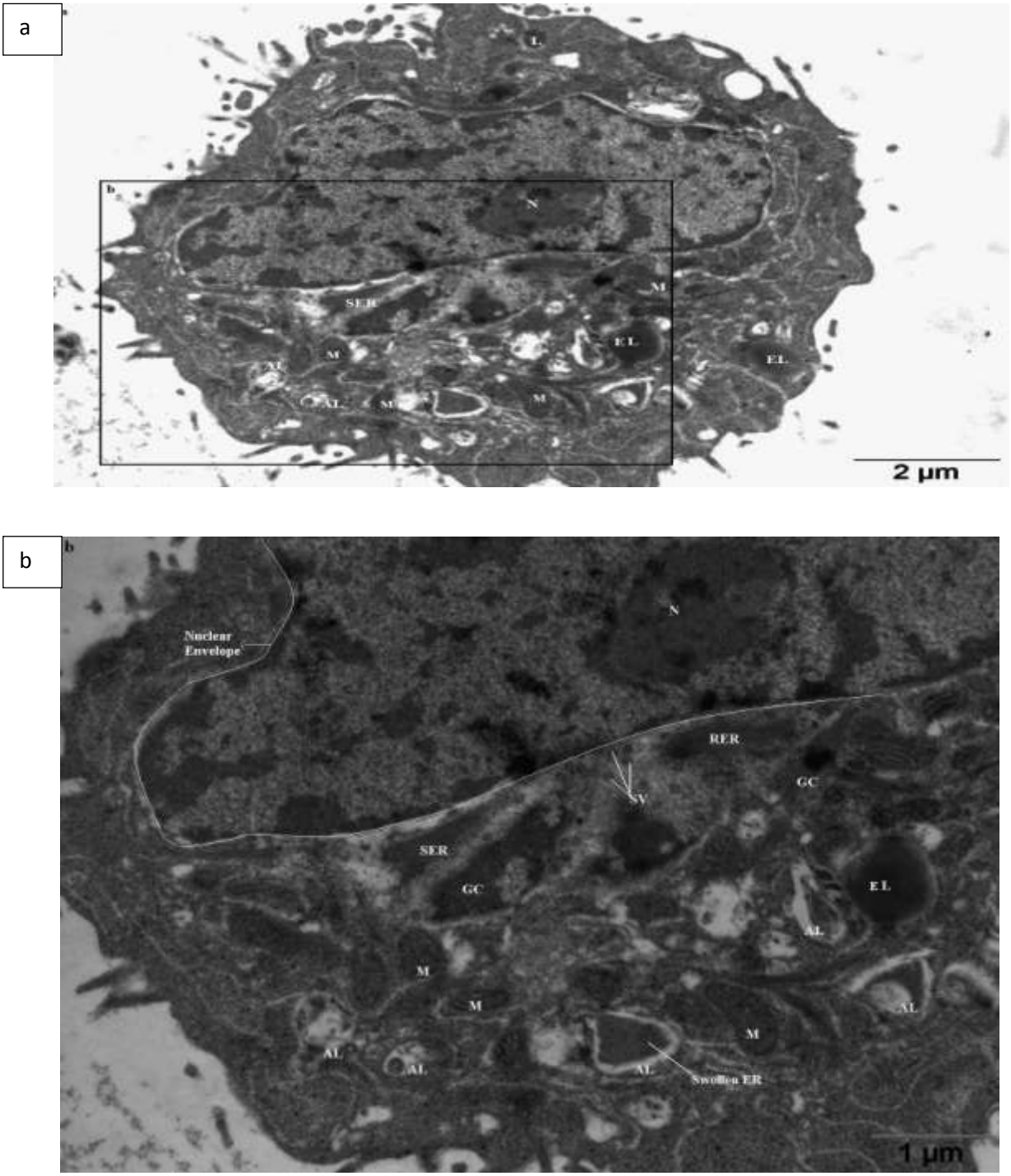


Figure 4.9 Transmission electron micrographs of the untreated A549 non-small cell lung cancer cells. (a) The whole cell magnified at 2 µm and (b) a section of (a) magnified at 1

μm. N, nucleolus; ER; endoplasmic reticulum; SER, smooth ER; RER, rough ER; GC, Golgi complex, sv, small vesicle; EL, early lysosome; AL, autolysosome; M; mitochondria.

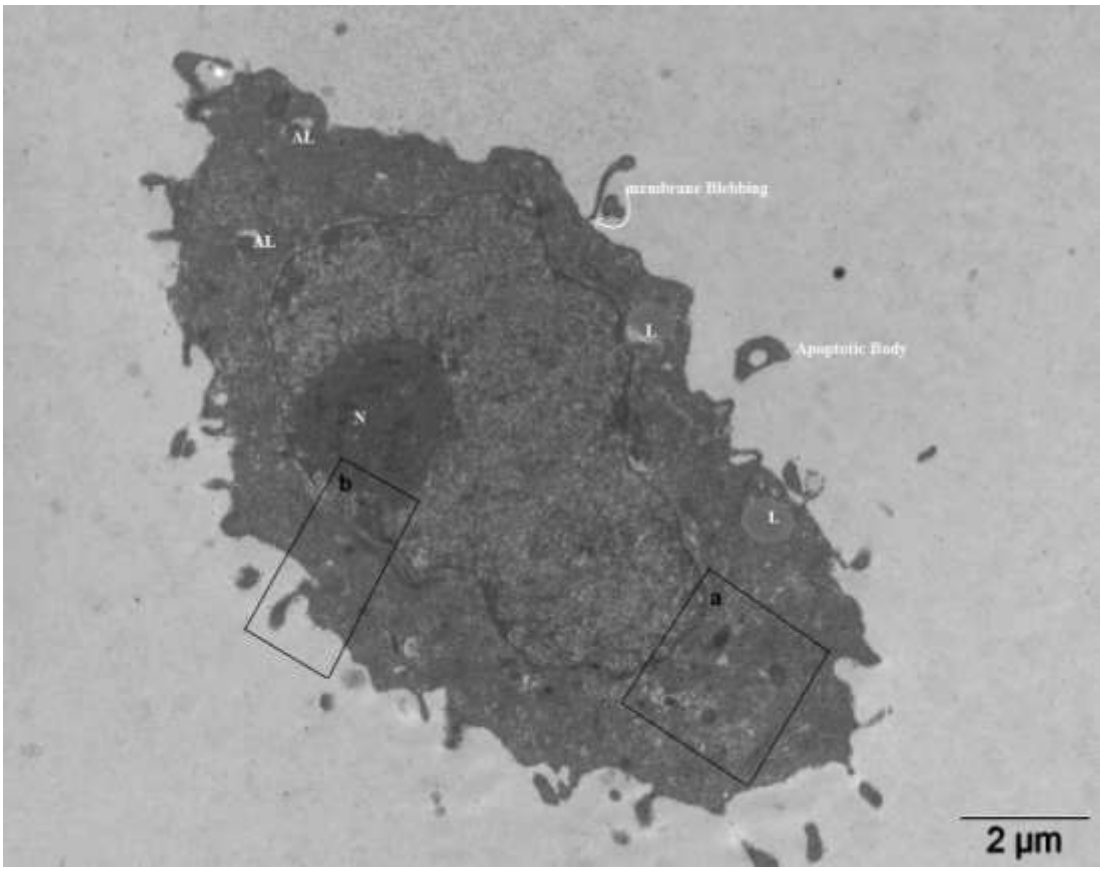
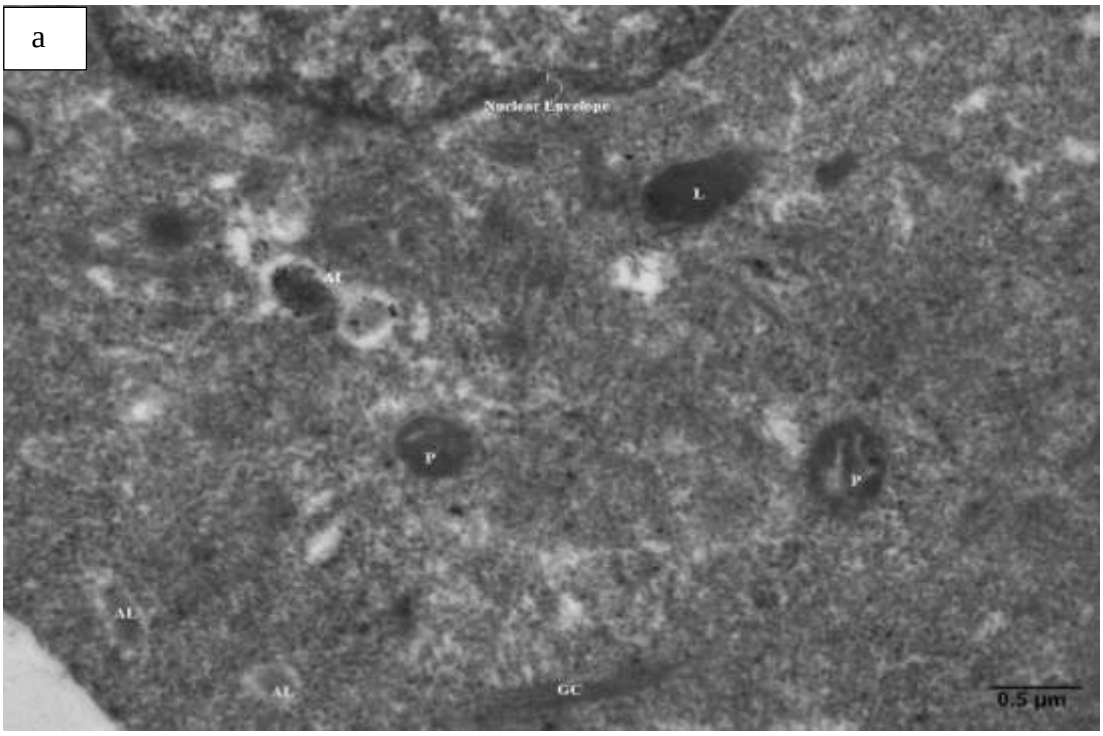


Figure 4.10 Transmission electron micrographs showing early signs of apoptosis of A549 non-small cell lung cancer cells treated with GEM10, incubated for 48 h. (a) The whole cell magnified at 2 μm and sections (a) and (b) were enlarged in Figure 4.20 at magnification of 0.5 μm. N, nucleolus; L, lysosome; AL, autolysosome.

a



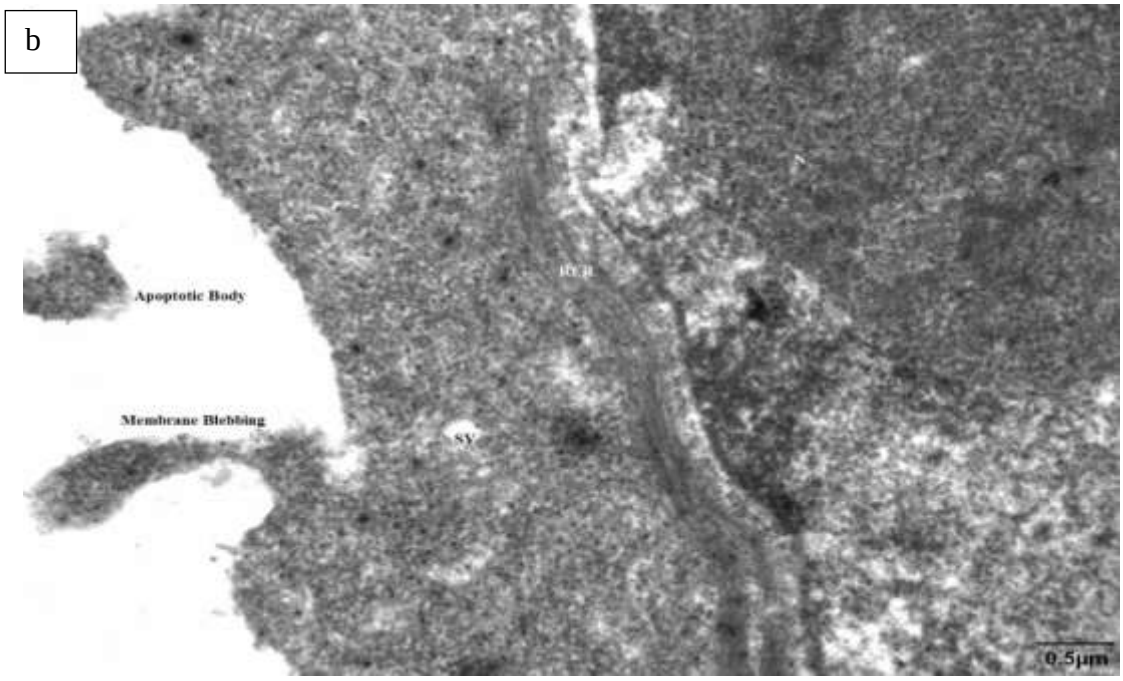


Figure 4.11 Transmission electron micrographs of A549 non-small cell lung cancer cells treated with GEM10, incubated for 48 h. Both (a) and (b) are sections of Figure 4.19 were magnified at 0.5 µm. ER; endoplasmic reticulum; RER, rough ER;GC, Golgi complex; L, lysosome; AL, autolysosome; P, peroxisomes.

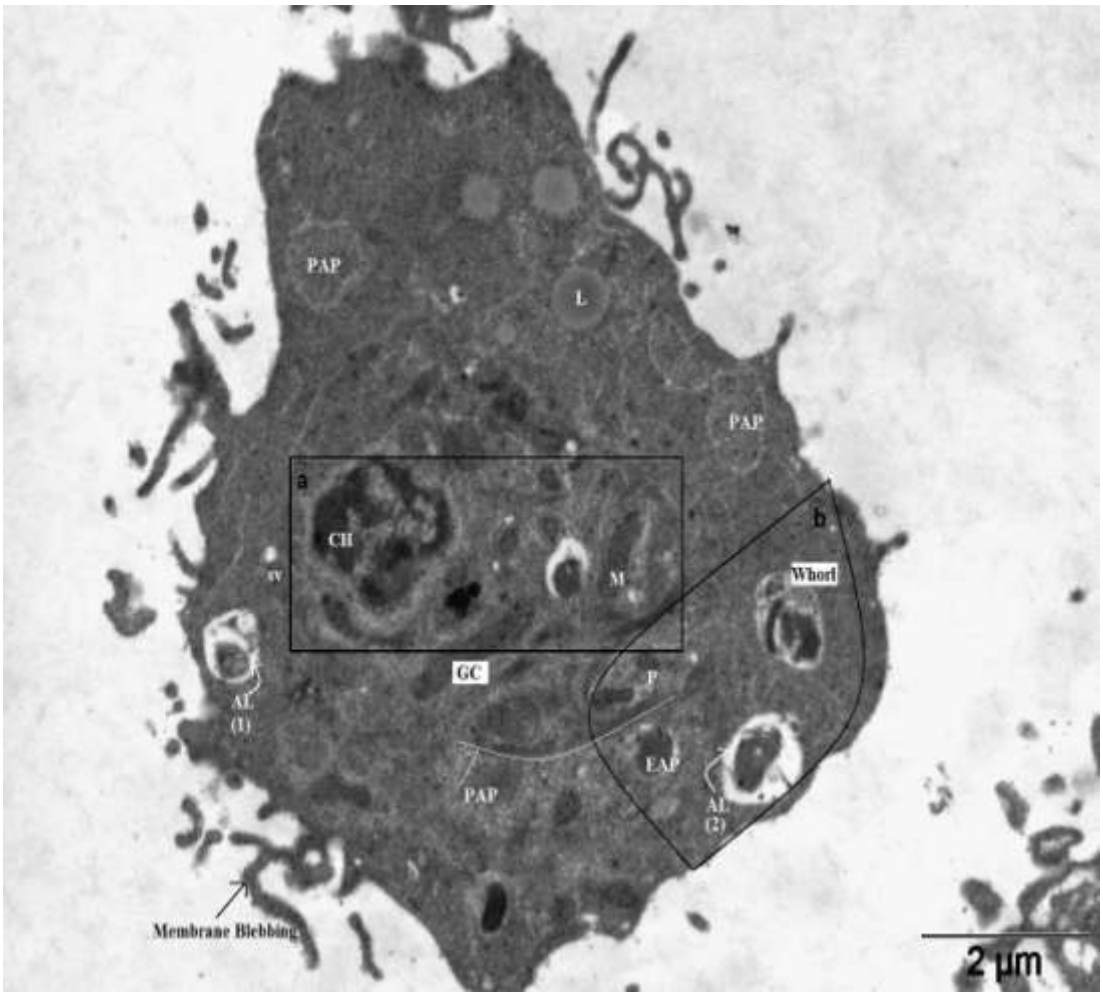


Figure 4.12 Transmission electron micrograph showing the late signs of apoptosis of A549 non-small cell lung cancer cells treated with GEM10, incubated for 48 h. (a) The whole cell magnified at 2 µm and sections (b) and (c) were magnified in Figure 4.22 at 1 and 0.5 µm, respectively. CH: chromatid fragments; PAP: pre-autophagosome, EAP: early autophagosome; L, lysosome; AL, autolysosome; AL(1), includes swollen mitochondria and endoplasmic reticulum; AL(2), includes swollen mitochondria; GC, Golgi complex; M, mitochondria; sv, small vesicle; P, peroxisomes; Whorl, large degradative endosome.

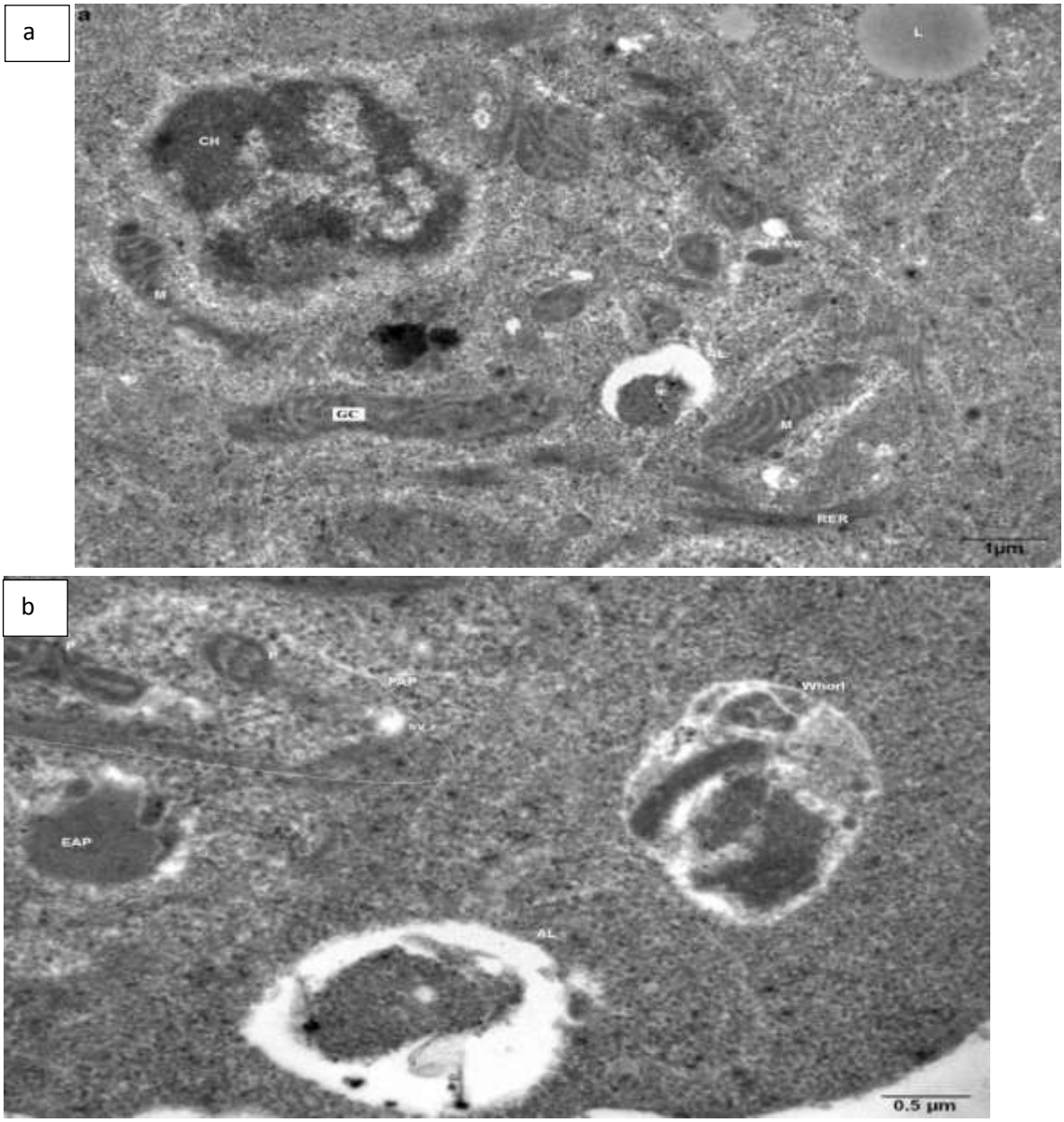


Figure 4.13 Transmission electron micrographs showing the late signs of apoptosis of A549 non-small cell lung cancer cells treated with GEM10, incubated for 48 h. Both (a) and (b) are sections of Figure 4.21 were magnified at 1 and 0.5 µm. CH: chromatid fragments; PAP: pre-autophagosome, EAP: early autophagosome; L, lysosome; AL, autolysosome includes swollen mitochondria; GC, Golgi complex; M, mitochondria; P, peroxisomes; Whorl, large degradative endosome; sv, small vesicle; RER, rough endoplasmic reticulum.

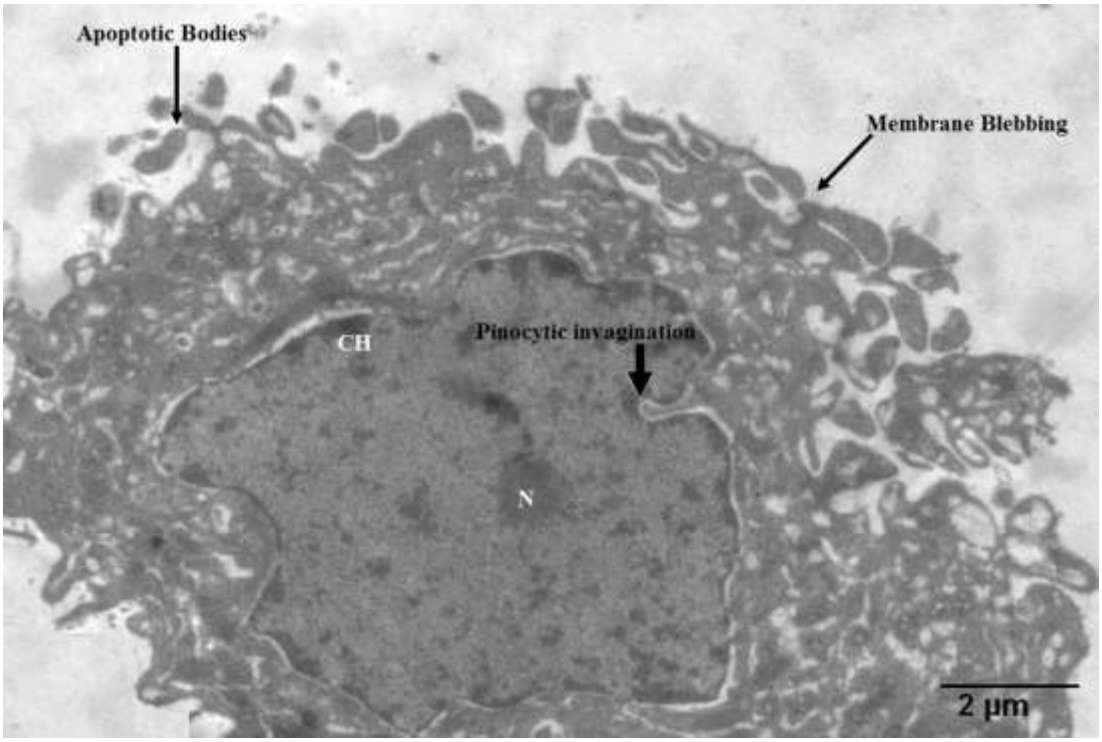


Figure 4.14 Transmission electron micrographs showing the late signs of apoptosis and extensive membrane blebbing of A549 non-small cell lung cancer cells treated with GEM10, incubated for 48 h. The whole cell was magnified at 2 µm. CH, chromatid fragments; N, shrunken nucleolus.

Few A549 cells, treated with MEb10+, were ultrastructurally visualized. One of the cells displayed late signs of apoptosis as the nucleolus disappeared, leaving few chromatid fragments (Figure 4.24). Further, the apoptosis integrated obviously with the autophagy pathway as clearly elaborated by magnifying the upper corner section of Figure 4.24 and displayed in Figure 4.25 which exhibited the development of multivesicle bodies (MVBs), AL's, Whorl, EAP's, condensed M's, membrane blebbing, pinocytic invagination and apoptotic bodies.

Another cell, treated with MEb10+, have showed early signs of apoptosis as the nucleolus started to fragment, pinocytic invagination was initiated, and EAP, AL's and membrane blebbing were formed (Figure 4.26). Additionally, one of the obtained cells has undergone mitotic cell division as exhibited in Figure 4.27, and clearly showed endosomal digestion of the nucleolus, condensed M and few L's, AL's, large vesicles (lv's) and Whorl. On the other hand, cells treated with MEb10 became ghost cells as the nucleus and all organelles vanished (Figure 4.28).

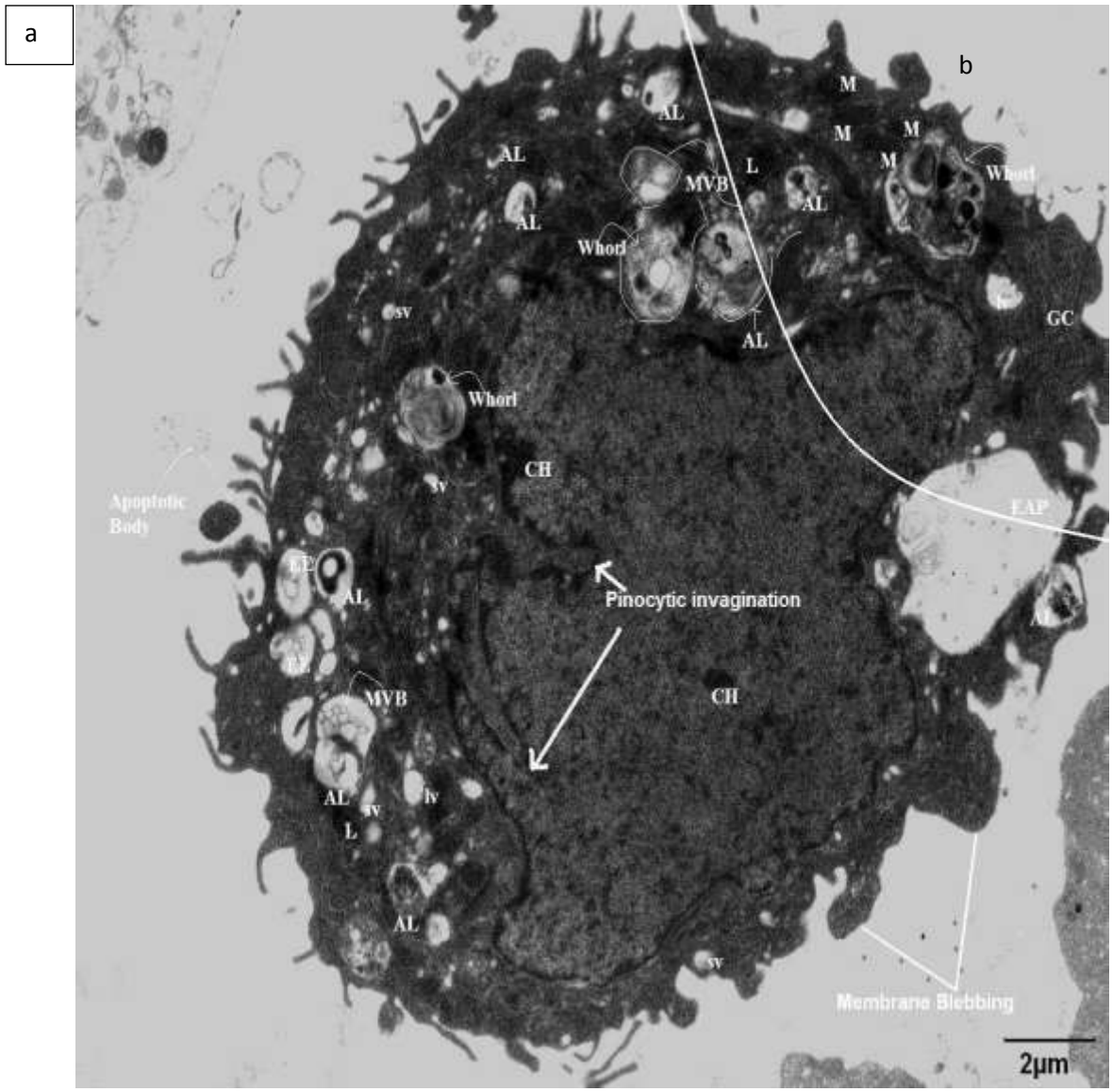


Figure 4.15 Transmission electron micrographs showing the late signs of apoptosis of A549 non-small cell lung cancer cells treated with MEb10+, incubated for 48 h. (a) The whole cell was magnified at 2 µm and section (b) was magnified at 1 µm in Figure 4.25. CH: chromatid fragments; L, lysosome; AL, autolysosome; GC, Golgi complex; M, mitochondria;Whorl, large degradative endosome; sv, small vesicle; MVB, multivesicle bodies; EAP, early autophagosome; lv, large vesicle.

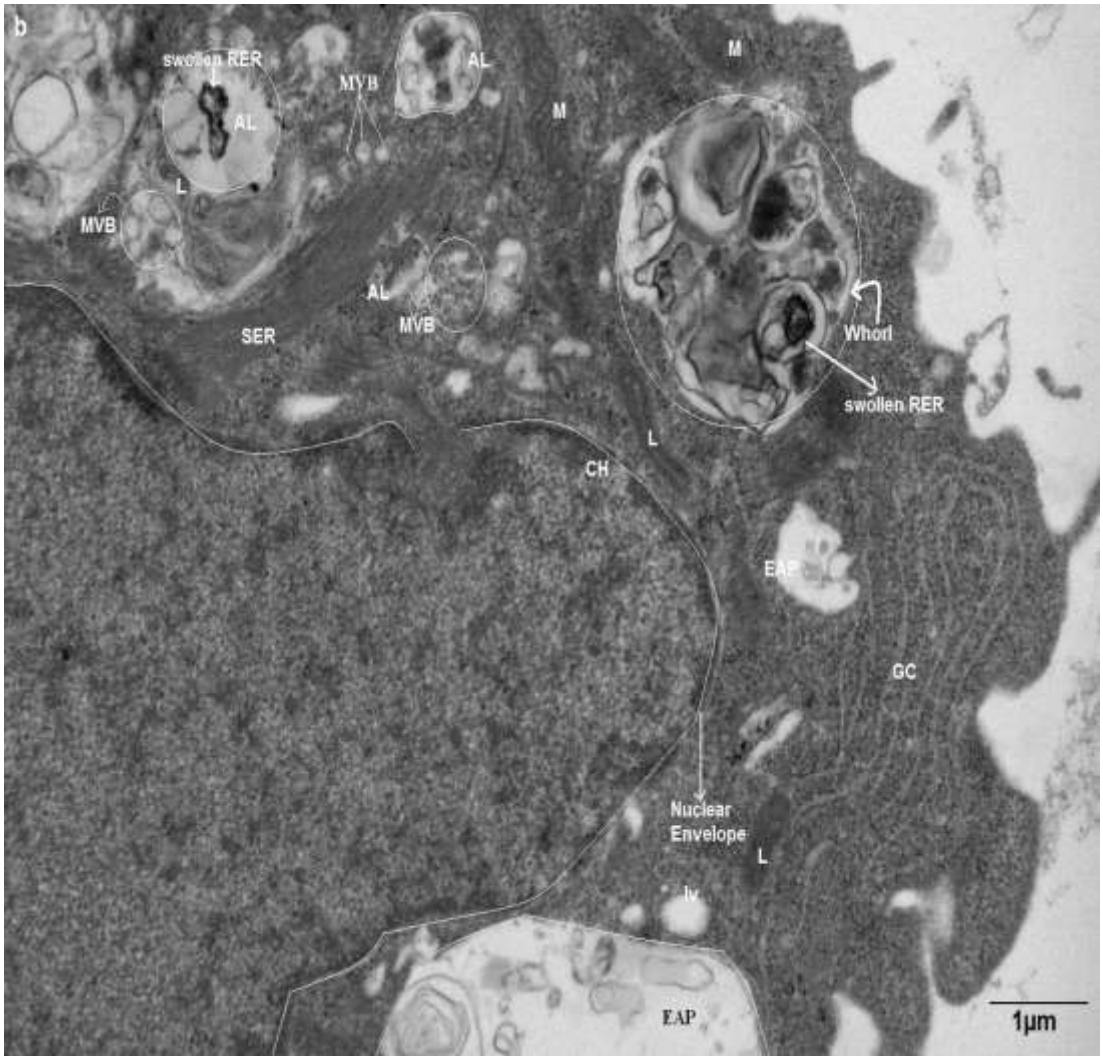


Figure 4.16 Transmission electron micrograph of section (b) of Figure 4.24 showing the autophagocytosis of A549 non-small cell lung cancer cells treated with MEb10+, incubated for 48 h, magnified at 1 µm. CH: chromatid fragments; L, lysosome; AL, autolysosome; ER, endoplasmic reticulum; RER, rough ER; SER, smooth ER; GC, Golgi complex; M, mitochondria; Whorl, large degradative endosome; lv, large vesicle; MVB, multivesicle bodies;

EAP, early autophagosome.

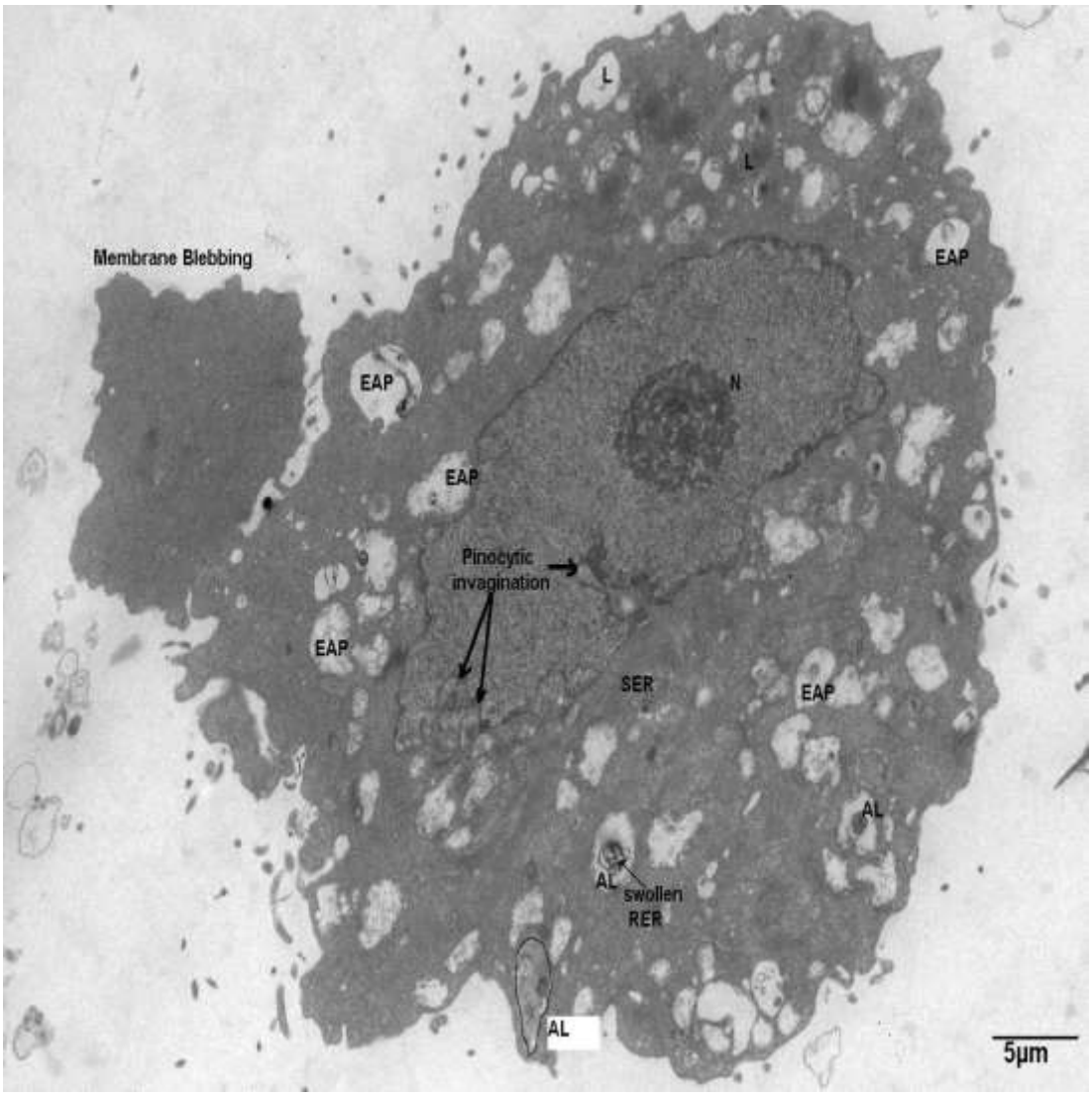
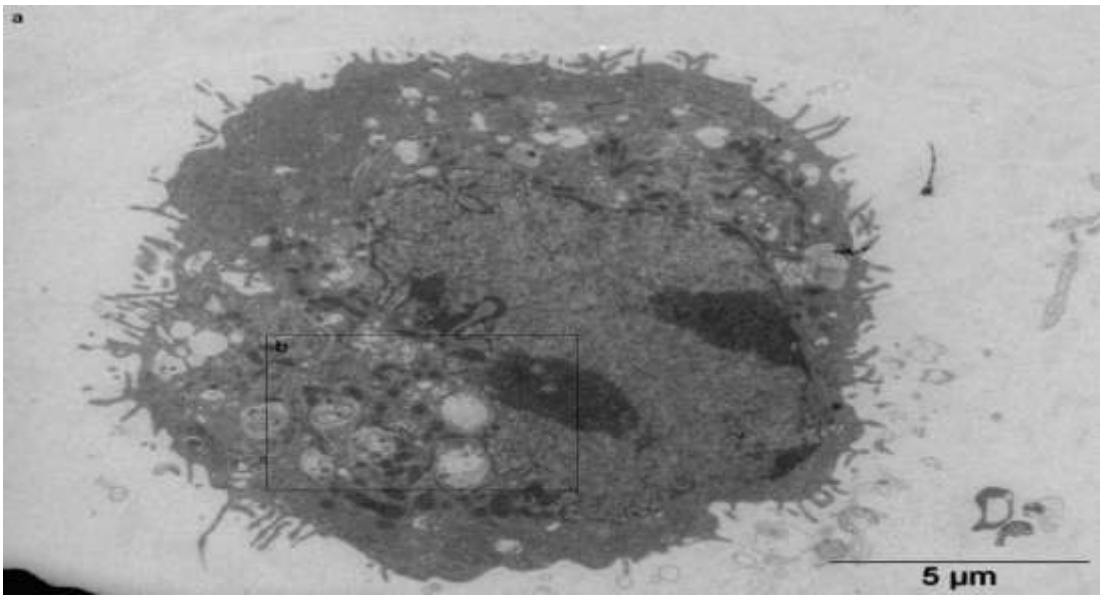


Figure 4.17 Transmission electron micrograph showing early signs of apoptosis of A549 non-small cell lung cancer cells treated with MEb10+, incubated for 48 h, magnified at 5 µm. N: nucleolus; L, lysosome; AL, autolysosome; ER, endoplasmic reticulum; RER, rough ER; SER, smooth ER; EAP, early autophagosome.

a



b

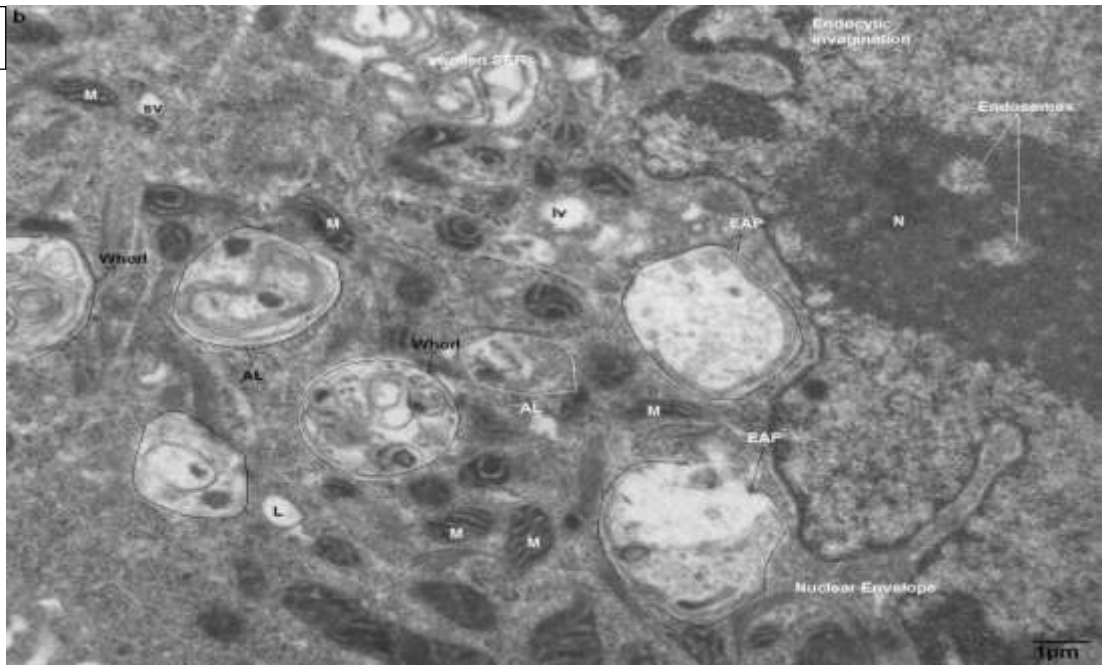


Figure 4. 18 Transmission electron micrographs showing early signs of apoptosis of A549 non-small cell lung cancer cells undergoing cell division, treated with MEb10+, incubated for 48 h. (a) the whole cell magnified at 5 µm and (b) section of the whole cell (a) magnified at 1 µm. N: nucleolus; L, lysosome; AL, autolysosome; SER, smooth endoplasmic

reticulum; M, mitochondria; Whorl, large degradative endosome; sv, small vesicle; lv, large vesicle; EAP, early autophagosome.

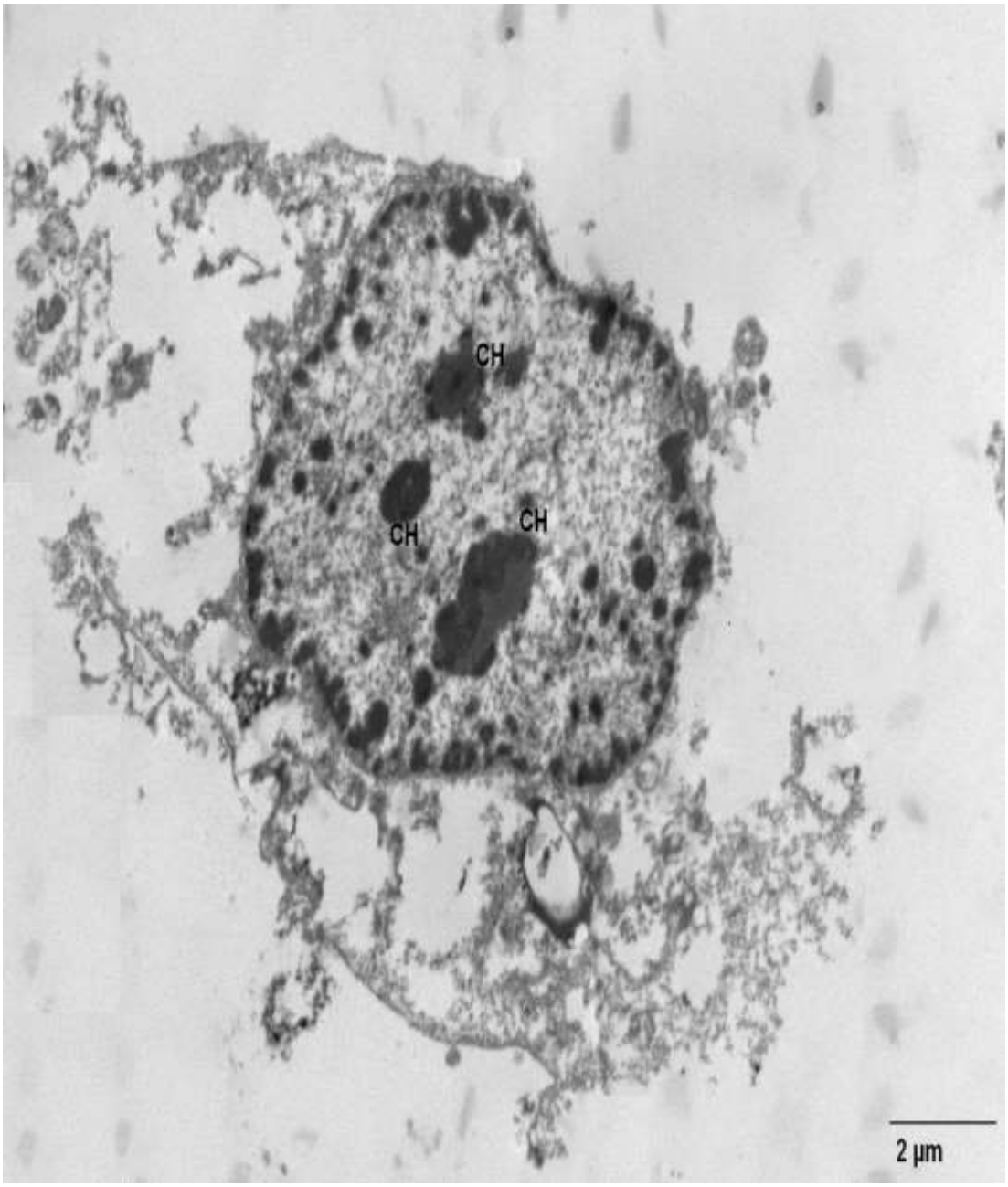


Figure 4.19 Transmission electron micrographs showing ghost cell of A549 non-small cell lung cancer cell, treated with MEb10, incubated for 48 h. CH: