CHAPTER IV

RESULTS AND DISCUSSION

Part 1. Effects of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and its active constituent (oxyresveratrol) on fibroblast proliferation and cytotoxicity

1.1 Proliferation assay

The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan was solubilized by the addition of a detergent and quantified spectrophotometrically to determine the extent of proliferation.

Before the MTT assay, a standard calibration curve between the number of fibroblasts and the optical density (OD) at 595 nm was constructed to ensure the validity of the test. As seen from the data in Table 2 and Figure 12, the standard curve was linear in the range of 0 to 20,000 cells with the coefficient of determination (R^2) of 0.9852 (Table 3).

Table 2 Data of standard curve between absorbance (OD) and cell number

Cell number (x)	OD1	OD2	OD3	average OD (y)
0	0.075	0.076	0.076	0.076
500	0.081	0.081	0.080	0.081
1000	0.084	0.083	0.084	0.084
4000	0.123	0.132	0.122	0.126
7000	0.194	0.177	0.198	0.190
10000	0.260	0.220	0.215	0.232
15000	0.292	0.280	0.294	0.289
20000	0.377	0.367	0.341	0.362

Figure 12 Standard curve from the relationship between OD and cell number

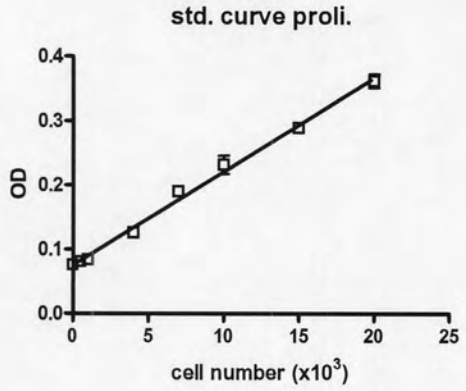


Table 3 Data were interpolated from linear regression

Best-fit values	
Slope	0.00001456 ± 0.0000003800
Y-intercept	0.07501 ± 0.003779
X-intercept	-5152
1/slope	68680
95% Confidence Intervals	
Slope	0.00001377 to 0.00001535
Y-intercept when X=0.0	0.06717 to 0.08285
X-intercept when Y=0.0	-6118 to -4258
Goodness of Fit	
R^2	0.9852
Sy.x	0.0128
Is slope significantly non-zero?	
F	1468
DFn, DFd	1.000, 22.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	8
Maximum number of Y replicates	3
Total number of values	24
Number of missing values	0

Equation $y = 0.00001456x + 0.07501$

$r^2 = 0.9852$

Table 4 shows the absorbance and number of viable fibroblast cells after treatment with Puag-Haad or oxyresveratrol at various concentrations (10, 25 and 50 μg/mL) in comparison with epigallocatechin gallate or EGCG (the active component of green tea), French pine bark extract, L-ascorbic acid (Vitamin C) or Trolox® (water soluble form of vitamin E) at the same concentrations. As the concentration was increased, proliferation of fibroblasts also increased for all antioxidants, especially at concentrations of 25 and 50 μg/mL.

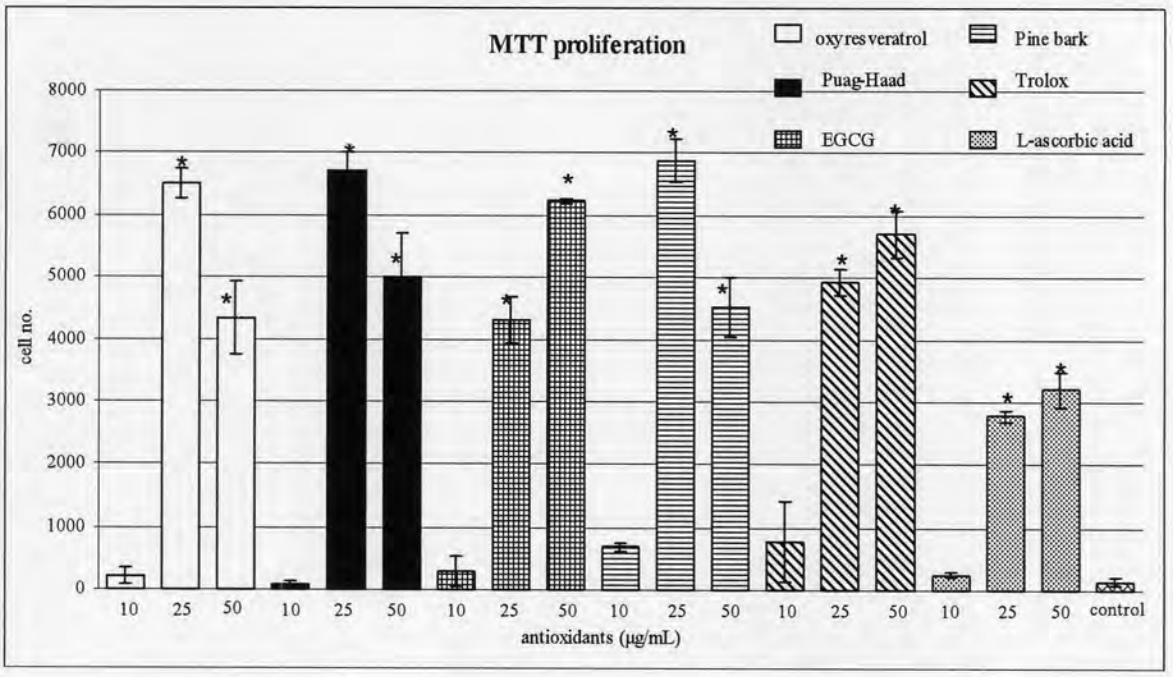
The data on the number of viable cells were analysed by one-way analysis of variance (ANOVA) at 5% significance level. Significant differences were found among all the antioxidants studied ($P < 0.05$). Subsequent post-ANOVA Dunnett's test at the same significance level revealed that there were significant differences in the number of viable fibroblasts between the control (cells with no antioxidant added) and each of the antioxidant-treated groups at concentrations 25 and 50 μg/mL. The results thus suggested that these two concentrations were effective in stimulating fibroblast proliferation. On the other hand, the number of viable cells treated with 10 μg/mL antioxidant increased only slightly for all antioxidant-treated groups (except Puag-Haad which slightly decreased) and did not differ statistically from the control ($P > 0.05$, Dunnett's test), indicating that this concentration was too low to stimulate any significant changes in fibroblast proliferation.

Table 4 OD values and number of viable cells interpolated from the standard curve following incubation of fibroblast cells in the presence of various antioxidants at 0, 10, 25 and 50 μg/ml

sample		Conc. (μg/mL)	OD1	OD2	OD3	cell no.1	cell no. 2	cell no.3	Average	S.D.
Oxyresveratrol	72 hr	10	0.076	0.080	0.078	68.0	342.7	205.4	205.4	137.4
		25	0.170	0.166	0.173	6524.0	6249.3	6730.1	6501.1	241.2
		50	0.137	0.130	0.147	4257.6	3776.8	4944.4	4326.2	586.8
Puag-Haad		10	0.076	0.077	0.076	68.0	136.7	68.0	90.9	39.7
		25	0.173	0.177	0.168	6730.1	7004.8	6386.7	6707.2	309.7
		50	0.138	0.148	0.158	4326.2	5013.0	5699.9	5013.0	686.8
EGCG		10	0.076	0.079	0.083	68.0	274.0	548.8	296.9	241.2
		25	0.138	0.143	0.132	4326.2	4669.6	3914.1	4303.3	378.3
		50	0.166	0.165	0.166	6249.3	6180.6	6249.3	6226.4	39.7
Pine bark		10	0.086	0.085	0.084	754.8	686.1	617.4	686.1	68.7
		25	0.170	0.180	0.175	6524.0	7210.9	6867.4	6867.4	343.4
		50	0.149	0.138	0.136	5081.7	4326.2	4188.9	4532.3	480.8
Trolox®		10	0.081	0.097	0.081	411.4	1510.3	411.4	777.7	634.5
		25	0.144	0.150	0.146	4738.3	5150.4	4875.7	4921.5	209.8
		50	0.159	0.163	0.152	5768.5	6043.3	5287.8	5699.9	382.4
L-ascobic acid		10	0.078	0.079	0.079	205.4	274.0	274.0	251.1	39.7
		25	0.115	0.114	0.117	2746.6	2677.9	2883.9	2769.5	104.9
		50	0.126	0.121	0.118	3502.1	3158.7	2952.6	3204.4	277.6
control		0	0.076	0.077	0.078	68.0	136.7	205.4	136.7	68.7

Note: each well was originally seeded with 4,000 fibroblast cells

Figure 13 Histogram comparing the number of viable cells following incubation of fibroblasts with different antioxidants at 0, 10, 25 and 50 μg/ml
(data = mean ± SD, n = 3)



Note: each well was originally seeded with 4,000 fibroblast cells
* $P < 0.05$ when compared to control (no antioxidant) using Dunnett's test

Exposure of fibroblasts to oxyresveratrol increased the number of viable cells from control (136.68 ± 68.68 cells) to 205.36 ± 137.36, 6501.15 ± 241.20 and 4326.24 ± 586.81 cells at the concentrations of 10, 25 and 50 μg/mL, respectively, equivalent to 1.5-, 47.6- and 31.7-fold increase over control. Puag-Haad also gave proliferative effect profile similar to oxyresveratrol, especially at concentrations of 25 and 50 μg/mL, at which the number of viable cells respectively increased to 6707.19 ± 309.70 and 5013.05 ± 686.81 cells equivalent to 49.1 and 36.7 folds over control. At 10 μg/mL, the number of viable cells slightly decreased to 90.89 ± 39.65 cells, equivalent to 0.67 folds of control. However, the values were not statistically different from control ($P > 0.05$, Dunnett's test). Therefore, the data further substantiated previous observations with other antioxidants that this concentration might be too low to exert any detectable changes in fibroblast proliferation using this method.

From the data in Table 4 and Figure 13, EGCG showed a proliferative effect on fibroblasts with an increase in the number of viable cells of 2.2, 31.5 and 45.6 folds over control at antioxidant concentrations of 10, 25 and 50 μg/mL, respectively. At the same respective concentrations, French pine bark extract increased the number of viable cells by 5.0, 50.2 and 33.2 folds whereas Trolox® increased the proliferation by 5.7, 36.0 and 41.7 folds and L-ascorbic acid increased by 1.8, 20.3 and 23.4 folds. It is interesting to note that the maximum proliferative effect exerted by EGCG, pine bark extract and Trolox® was in the same magnitude as Puag-Haad and oxyresveratrol (about 40 – 50 folds increase over control). On the other hand, L-ascorbic showed the lowest proliferative extent, with maximum increase of only about 20 folds. Histograms comparing the number of viable fibroblasts among different antioxidants at the same concentration are also provided in Figure 14. From this figure, Puag-Haad and oxyresveratrol demonstrate a proliferative effect comparable to EGCG, pine bark extract and Trolox® whereas L-ascorbic acid seems to have a relatively smaller effect.

Also, it should be noticed that the number of viable fibroblasts in the control group at the time of MTT measurements was 136.68 ± 68.68 cells, which was lower than the number of cells originally placed in the plate, i.e., 4,000 freshly trypsinized fibroblasts per well. The OD values of the control wells were also lower than that of the standard curve seeded at 4000 cells (Table 2). This could be due to the difference in the time of placing cells on the plate as well as the different incubation conditions between the standard and the control wells. Cells for the standard curve were incubated for only 24 hrs. before the MTT analysis whereas all the cells in the control and the antioxidant-treated wells were subjected to several times of incubation and reincubation, with several changes of medium and temperature occurring in between. Thus, the integrity of the fibroblasts in the control and the antioxidant-treated wells could be more compromised than the intact cells of the standard curve, resulting in smaller OD and number of viable cells than the latter.

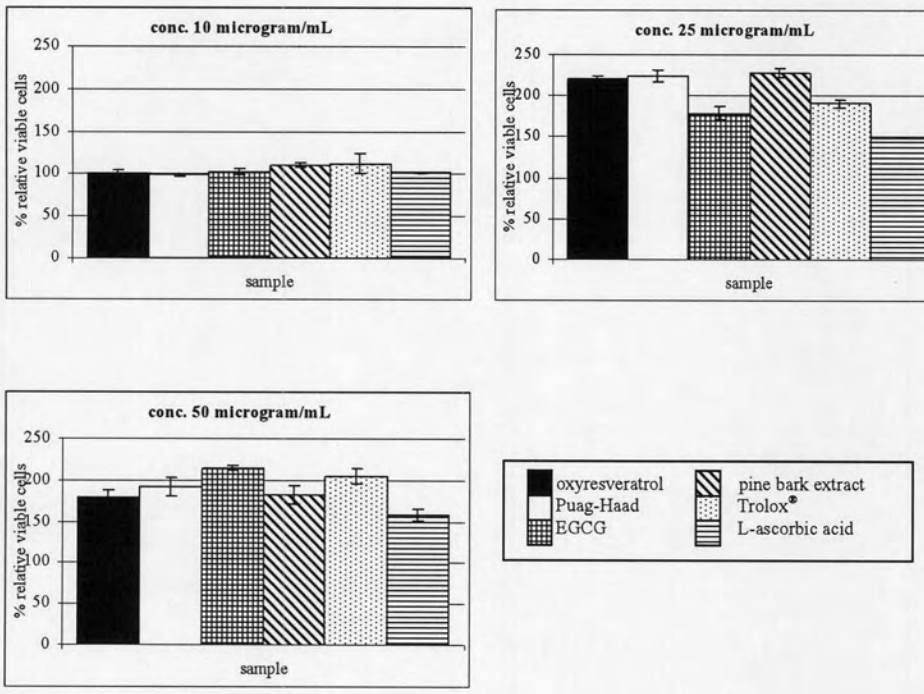
This effect was similarly observed at the lowest antioxidant concentration (10 μg/mL), at which the concentration was too low to stimulate significant proliferation, resulting in the final viable cells of all antioxidant-treated groups also being much smaller than 4,000 cells (Table 4). Nevertheless, this had no deleterious outcome on the validity of comparison between the control and the antioxidant-treated groups

since both were subjected to exactly the same experimental conditions, i.e., the same total incubation time and frequency of medium changing.

Also in this study, only blank for the standard curve was performed, in which only the medium (no cells) was added to the assay well. Separate blanks for control and sample (antioxidant-treated) groups were not prepared because their OD values were assumed to be similar to the standard blank. However, their OD values could in fact be different because the total incubation time as well as the frequency of removing old and adding new medium was greater for the control and the sample blank than for the standard blank. The possible residual amount of DMEM and FBS remaining in the well following each removal of the old medium may have contributed to the difference in the OD values. This implied that the use of the blank OD intended for the standard to correct for the OD values of the control and the samples might not be appropriate. Therefore, blank correction of the OD values was omitted in this study to minimize data variation.

It can be concluded from this part that all investigated antioxidants had a significant proliferative effect on fibroblasts, especially at the concentrations of 25 and 50 μg/mL. As none of the antioxidants gave a significantly lower OD values than the control group, it also can be stated that all the studied antioxidants had no cytotoxic effect on fibroblasts in the concentration range of 10 – 50 μg/mL.

Figure 14 Histograms comparing percentage of viable fibroblasts relative to control (100%) among different antioxidants (Data = mean ± SD, n = 3)



1.2 Cytotoxic Assay

The cytotoxicity of Puag-Haad and reference antioxidants was evaluated using the same MTT assay by further increasing the antioxidant concentration to determine if it had any negative effect on the viable cells.

Similar to the proliferation study, a standard curve between the number of fibroblasts and the optical density (OD) at 595 nm was also constructed. As seen from the data in Table 5 and Figure 15, the standard curve was linear in the range of 0 to 20,000 cells with the coefficient of determination (R^2) of 0.9951 (Table 6).

Table 5 Data of standard curve between absorbance (OD) and cell number

Cell number (x)	OD1	OD2	OD3	average OD (y)
0	0.114	0.113	0.115	0.114
500	0.117	0.118	0.116	0.117
1000	0.126	0.120	0.122	0.123
4000	0.134	0.133	0.134	0.134
10000	0.160	0.159	0.163	0.161
15000	0.180	0.186	0.182	0.183
20000	0.200	0.207	0.206	0.204

Figure 15 Standard curve from the relationship between OD and cell number

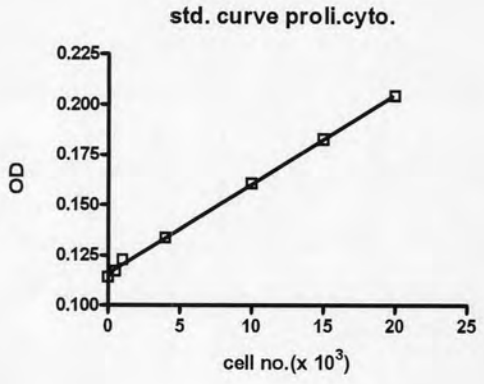


Table 6 Data were interpolated from linear regression

Best-fit values	
Slope	0.000004452 ± 0.00000007171
Y-intercept	0.1157 ± 0.0007385
X-intercept	-26000
1/slope	224600
95% Confidence Intervals	
Slope	0.000004301 to 0.000004602
Y-intercept when X=0.0	0.1142 to 0.1173
X-intercept when Y=0.0	-27240 to -24840
Goodness of Fit	
R^2	0.9951
Sy.x	0.002415
Is slope significantly non-zero?	
F	3853
DFn, DFd	1.000, 19.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	7
Maximum number of Y replicates	3
Total number of values	21
Number of missing values	0

Equation $y = 0.000004452x + 0.1157$

$r^2 = 0.9951$

Table 7 shows the absorbance and number of viable fibroblasts after treatment with Puag-Haad or oxyresveratrol in comparison with EGCG, French pine bark extract, L-ascorbic acid or Trolox® at 25, 100 and 250 μg/mL. At 25 μg/mL, all antioxidants showed a significant proliferative effect over the control group as previously observed ($P < 0.05$, one-way ANOVA and Dunnett's test). As the concentration was increased to 100 μg/mL, the number of viable cells decreased in all cases. However, proliferative effect was still present, particularly in cells treated with Puag-Haad, oxyresveratrol, EGCG or pine bark extract, with the number of viable cells significantly higher than control ($P < 0.05$). The cells treated with 100 μg/mL Trolox® or L-ascorbic acid also decreased from those at 25 μg/mL but the values did

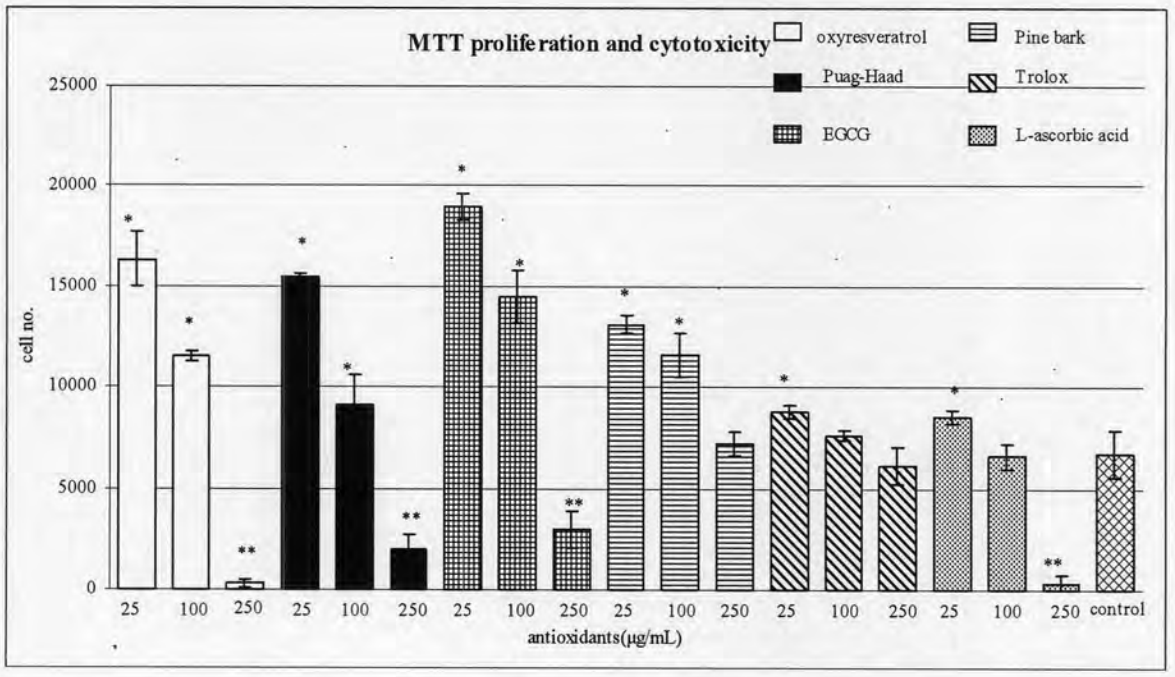
not differ from control ($P > 0.05$), suggesting the lack of proliferation. When the concentration of Puag-Haad, oxyresveratrol, EGCG and L-ascorbic acid was further increased to 250 μg/mL, the number of viable cells considerably dropped and became significantly smaller than control ($P < 0.05$), indicating a significant cytotoxic effect. On the other hand, the number of viable cells treated with either 250 μg/mL of pine bark extract or Trolox® was not different from control ($P > 0.05$), implying that these two antioxidants may have a lower cytotoxic effect than the rest of the studied antioxidants.

Table 7 OD values and number of viable cells interpolated from the standard curve of the MTT assay following incubation of fibroblast cells in the presence of various antioxidants at 0, 25, 100 and 250 μg/mL

sample		Conc. (μg/mL)	OD1	OD2	OD3	cell no.1	cell no. 2	cell no.3	Average	S.D.
Oxyresveratrol	72 hr	25	0.195	0.187	0.183	17812.2	16015.3	15116.8	16314.8	1372.4
		100	0.168	0.167	0.166	11747.5	11522.9	11298.3	11522.9	224.6
		250	0.116	0.117	0.118	67.4	292.0	516.6	292.0	224.6
Puag-Haad		25	0.185	0.184	0.185	15566.0	15341.4	15566.0	15491.2	129.7
		100	0.161	0.149	0.159	10175.2	7479.8	9726.0	9127.0	1444.1
		250	0.127	0.121	0.126	2538.2	1190.5	2313.6	2014.1	722.0
EGCG		25	0.200	0.203	0.197	18935.3	19609.2	18261.5	18935.3	673.9
		100	0.187	0.176	0.178	16015.3	13544.5	13993.7	14517.8	1316.1
		250	0.129	0.133	0.125	2987.4	3885.9	2088.9	2987.4	898.5
Pine bark		25	0.172	0.176	0.174	12646.0	13544.5	13095.2	13095.2	449.2
		100	0.162	0.169	0.171	10399.8	11972.1	12421.4	11597.8	1061.5
		250	0.145	0.148	0.150	6581.3	7255.2	7704.4	7180.3	565.3
Trolox®		25	0.153	0.156	0.155	8378.3	9052.1	8827.5	8752.6	343.1
		100	0.149	0.149	0.151	7479.8	7479.8	7929.0	7629.5	259.4
		250	0.143	0.139	0.147	6132.1	5233.6	7030.5	6132.1	898.5
L-ascorbic acid		25	0.154	0.155	0.152	8602.9	8827.5	8153.6	8528.0	343.1
		100	0.144	0.148	0.143	6356.7	7255.2	6132.1	6581.3	594.3
		250	0.117	0.119	0.116	292.0	741.2	67.4	366.9	343.1
control		0	0.150	0.147	0.140	7704.4	7030.5	5458.2	6731.1	1152.7

Note: Each well was originally seeded with 4,000 fibroblast cells

Figure 16 Histogram comparing the number of viable cells following incubation of fibroblasts with different antioxidants at 0, 25, 100 and 250 μg/ml
(data = mean ± SD, n = 3)



Note: Each well was originally seeded with 4,000 fibroblast cells
* $P < 0.05$, Dunnett's test (greater than control or having proliferative effect)
** $P < 0.05$, Dunnett's test (less than control or having cytotoxic effect)

Oxyresveratrol gave number of viable cells of 16314.8 ± 1372.4 and 11522.9 ± 224.6 cells at concentrations 25 and 100 μg/mL of respectively, which were higher than control (6731.1 ± 1152.7 cells) by 2.4 and 1.7 folds. However, when the concentration was further increased to 250 μg/mL, the number of viable cells sharply dropped to 292.0 ± 224.6 cells, or 23 folds lower than control. At 25 and 100 μg/mL, Puag-Haad and EGCG also gave proliferative effect profile similar to oxyresveratrol, with the number of viable cells increasing from control to 15491.2 ± 129.7 (2.3-fold increase) and 9127.0 ± 1444.1 cells (1.4-fold increase) for Puag-Haad and to 18935.3 ± 673.9 (2.8-fold increase) and 14517.8 ± 1316.1 cells (2.2-fold increase) for EGCG, respectively. At 250 μg/mL, both Puag-Haad and EGCG also gave a significant drop

in the number of viable cells, with respective average values of 2014.1 $\pm$ 722.0 and 2987.4 $\pm$ 898.5 cells, or equivalent to 3.3- and 2.3-folds lower than control.

Pine bark extract, on the other hand, demonstrated no cytotoxic effect in the 25 – 250 μg/mL range. At 25 and 100 μg/mL, it gave significant proliferation, with a respective increase in the number of viable cells over control by 2 and 1.7 folds ($P < 0.05$, Dunnett's test). At 250 μg/mL, the number of viable cells also decreased but was not as sharply as Puag-Haad, oxyresveratrol and EGCG. Instead, the value was similar to control with the ratio close to 1 (1.1 folds over control; $P > 0.05$, Dunnett's test). Trolox® also showed no sign of cytotoxicity in the same concentration range. However, its proliferative effect was lower than the pine bark extract, about 1.3 folds over control at 25 μg/mL. At 100 and 250 μg/mL, the proliferative effect disappeared as the number of viable cells slightly dropped to that of control (about 1.1 folds over control; $P > 0.05$, Dunnett's test).

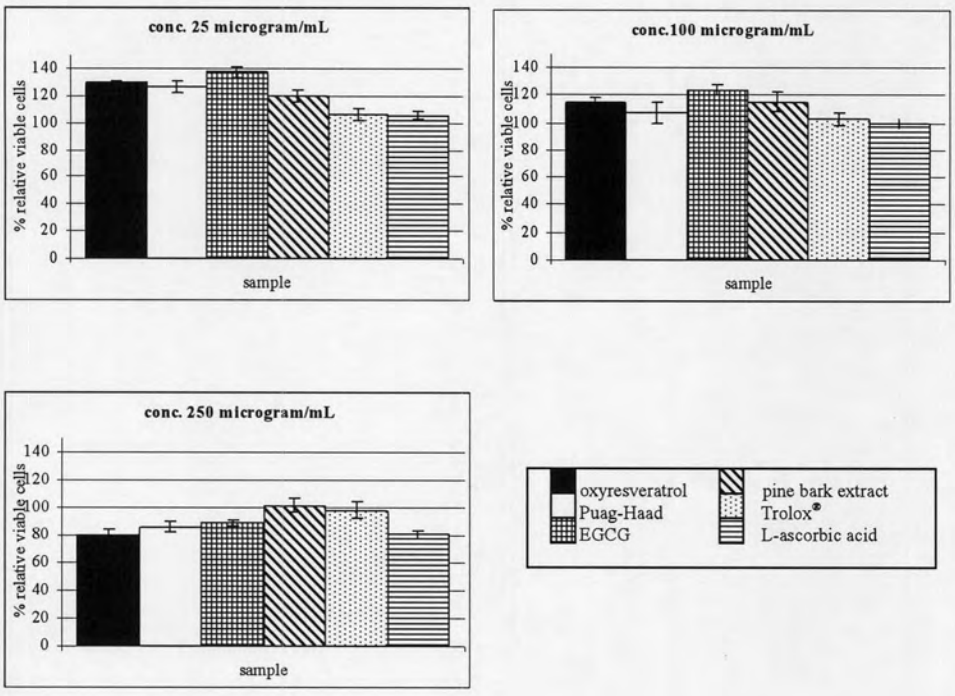
L-ascorbic acid demonstrated a moderate proliferative effect similar to Trolox® with significant proliferation at 25 μg/mL or about 1.3 fold increase over control ($P < 0.05$, Dunnett's test). However, as the concentration was increased, the proliferative effect disappeared and the number of viable cells became lower than that of control. At 250 μg/mL, a significant and marked cytotoxic effect was observed, at which the viable cells became 18.4 folds less than control ($P < 0.05$, Dunnett's test).

It is also interesting to note that the number of viable cells after MTT test of the control group was 6731.1 cells, which was greater than the originally seeded cells of 4,000. The result was quite opposite from the previous study in which the number of cells in the control group was only 136.7 cells. This difference was also observed in the values of cell number at 25 μg/mL concentration for all antioxidants between the two studies, in which the second study always yielded the higher number of cells. The reasons as to this discrepancy were not clearly known. However, different sources of human dermal fibroblast cells were used between the proliferation and cytotoxicity studies. The first study used the cells coded SIF 49 whereas the second study used the cells coded CC 2511. It is possible that different sources of fibroblasts and study periods may have an influence on the ability of the cells to proliferate and their susceptibility to antioxidants. Nevertheless, the data at 25 μg/mL concentration of antioxidant from the first study agreed quite well with the second study in that all

the tested antioxidants were capable of stimulating proliferation of fibroblasts, regardless of the source of cells.

From the data in Table 7 and Figure 16, the proliferative effect started to decline for all antioxidants as the concentration was increased from 25 μg/mL, indicating that the optimum proliferative concentration was about 25 μg/mL. At 250 μg/mL, the concentration became cytotoxic to the fibroblast cells in the case of Puag-Haad, oxyresveratrol, EGCG and L-ascorbic acid whereas the pine bark extract and Trolox© did not produce significant cytotoxicity at this concentration. Figure 17 shows histograms comparing the percentage of viable cells relative to control among different antioxidants. From this figure it can be seen that at 250 μg/mL Puag-Haad, oxyresveratrol and EGCG were slightly more cytotoxic than the pine bark extract and Trolox©. They also stimulated proliferation to a greater extent than the latter two antioxidants, especially at 25 μg/mL. L-ascorbic acid, on the contrary, produced only moderate proliferative effect yet appeared to be more cytotoxic than Puag-Haad and EGCG at 250 μg/mL. It should also be noticed that Puag-Haad produced smaller cytotoxic effect than oxyresveratrol, its active component, which had the extent of cytotoxicity similar to L-ascorbic acid.

Figure 17 Histograms comparing percentage of viable fibroblasts relative to control (100%) among different antioxidants (Data = mean ± SD, n = 3)



Part 2. Evaluation of the ability of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and oxyresveratrol in decreasing oxidative stress-induced cell damages

2.1 Hydrogen peroxide (H_2O_2) – induced cell damages

The protective effect of Puag-Haad and reference antioxidants against H_2O_2-induced cell damage was evaluated by exposure of the fibroblasts to the mixture of 2 mM H_2O_2 and various concentrations of antioxidants in DMEM for 2 hr. MTT test was also used to determine the number of surviving fibroblasts in each group.

Figure 18 shows a standard curve between the number of fibroblasts and the optical density (OD) at 595 nm. As seen from the data in Table 8 and Figure 18, the standard curve was linear in the range of 500 to 20,000 cells with the coefficient of determination (R^2) of 0.9945 (Table 9), indicating the validity of the MTT test for this study.

Table 8 Data of standard curve between absorbance (OD) and cell number

Cell number (x)	OD1	OD2	OD3	average OD (y)
0	0.073	0.067	0.069	0.070
500	0.074	0.078	0.076	0.076
1000	0.083	0.087	0.079	0.083
4000	0.150	0.142	0.128	0.140
7000	0.206	0.219	0.213	0.213
10000	0.272	0.292	0.278	0.281
15000	0.361	0.373	0.366	0.367
20000	0.452	0.447	0.445	0.448

Figure 18 Standard curve from the relationship between OD and cell number

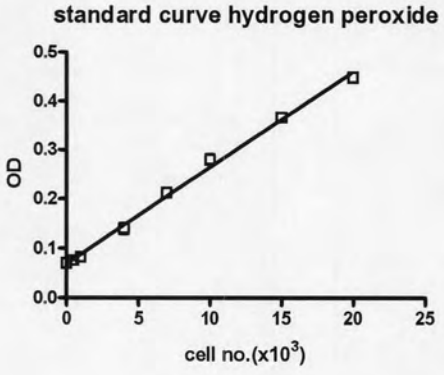


Table 9 Data were interpolated from linear regression

Best-fit values	
Slope	0.00001954 ± 0.0000003093
Y-intercept	0.06914 ± 0.003076
X-intercept	-3539
1/slope	51180
95% Confidence Intervals	
Slope	0.00001890 to 0.00002018
Y-intercept when X=0.0	0.06276 to 0.07552
X-intercept when Y=0.0	-4077 to -3024
Goodness of Fit	
R^2	0.9945
Sy.x	0.01042
Is slope significantly non-zero?	
F	3990
DFn, DFd	1.000, 22.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	8
Maximum number of Y replicates	3
Total number of values	24
Number of missing values	0

Equation $y = 0.00001954x + 0.06914$

$r^2 = 0.9945$

Table 10 shows the absorbance and number of viable fibroblasts remaining after 2-hr treatment with 2 mM H_2O_2 in the presence of Puag-Haad at 25, 50 and 100 μg/mL using MTT assay. The results were also compared to cells co-treated with H_2O_2 and other antioxidants (oxyresveratrol, EGCG, French pine bark extract, L-ascorbic acid or Trolox®) at the same concentrations as shown in Figure 19. Histograms comparing the percentage of viable fibroblasts relative to control (100 %) are also shown in Figure 20. It was found that all the antioxidants except L-ascorbic acid were able to provide protective effect against H_2O_2 at all concentrations studied as the number of viable fibroblasts was much greater than the control group, which was exposed to only H_2O_2. The untreated group, without exposure to H_2O_2 nor any antioxidant, naturally showed the highest number of viable fibroblasts (10774.1 $\pm$ 333.0 cells), which was close to the amount originally seeded.

Data on the number of viable cells exposed to H_2O_2 with and without antioxidants were then analyzed by one-way ANOVA at 5% level. Significant differences were found among the six antioxidants and the control group (treatment with only H_2O_2 without any antioxidant) ($P < 0.05$). Consequently, post-ANOVA Dunnett's test was further applied. There were significant differences between the control and each of the individual antioxidants in that the number of viable cells in the latter group was greater than the control, indicating the antioxidant's ability to protect the cells from H_2O_2–induced damages. This was especially the case with Puag-Haad, oxyresveratrol, EGCG, pine bark extract and Trolox®, for which the protective effect was found at all concentrations ($P < 0.05$; Dunnett's test). The values of viable cells in the untreated-group were not used in the ANOVA analysis because the basic experimental condition was different. However, visual comparison of Figure 19 revealed that this group yielded the highest number of viable fibroblasts since the cells were not exposed to H_2O_2.

Co-treatment of fibroblasts with L-ascorbic acid and H_2O_2, on the other hand, resulted in the protective effect observed only at 25 μg/mL concentration, where the number of surviving cells was significantly greater than control ($P < 0.05$; Dunnett's test). As the concentrations of L-ascorbic acid were increased to 50 and 100 μg/mL, the opposite effect was observed in which the number of surviving cells was significantly smaller than control ($P < 0.05$; Dunnett's test). This indicated that

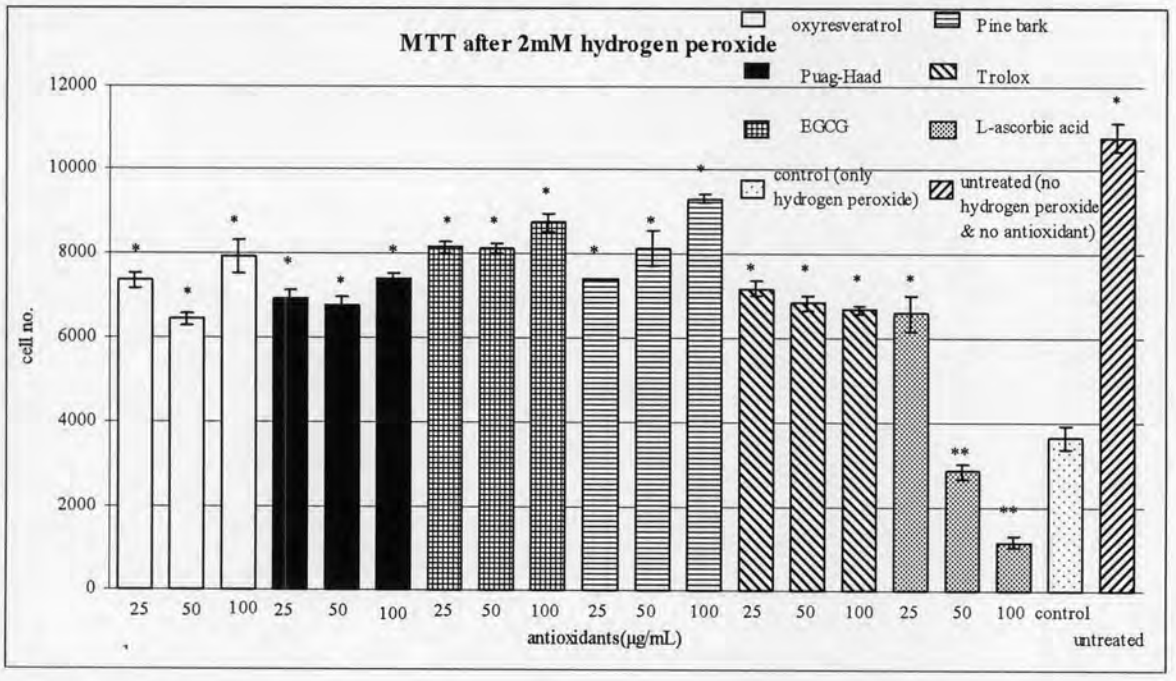
instead of having a protective effect against cell loss, higher concentration of L-ascorbic acid further intensified the damaging effect of H_2O_2.

Table 10 OD values and number of viable fibroblasts remaining after 2-hr treatment with mixture of 2 mM H_2O_2 and different antioxidants using MTT assay.

sample		Conc. (μg/mL)	OD1	OD2	OD3	cell no.1	cell no. 2	cell no.3	Average	S.D.
Oxyresveratrol	H_2O_2 2 mM	25	0.213	0.216	0.209	7362.3	7515.9	7157.6	7345.3	179.7
		50	0.195	0.192	0.197	6441.1	6287.6	6543.5	6424.1	128.8
		100	0.224	0.231	0.216	7925.3	8283.5	7515.9	7908.2	384.1
Puag-Haad		25	0.200	0.208	0.204	6697.0	7106.4	6901.7	6901.7	204.7
		50	0.201	0.197	0.205	6748.2	6543.5	6952.9	6748.2	204.7
		100	0.214	0.216	0.211	7413.5	7515.9	7260.0	7396.5	128.8
EGCG		25	0.228	0.231	0.225	8130.0	8283.5	7976.5	8130.0	153.5
		50	0.228	0.230	0.225	8130.0	8232.3	7976.5	8112.9	128.8
		100	0.235	0.242	0.242	8488.2	8846.5	8846.5	8727.1	206.8
Pine bark		25	0.213	0.213	0.214	7362.3	7362.3	7413.5	7379.4	29.5
		50	0.223	0.237	0.223	7874.1	8590.6	7874.1	8112.9	413.7
		100	0.253	0.251	0.249	9409.4	9307.1	9204.7	9307.1	102.4
Trolox®		25	0.211	0.211	0.205	7260.0	7260.0	6952.9	7157.6	177.3
		50	0.200	0.201	0.206	6697.0	6748.2	7004.1	6816.4	164.5
		100	0.201	0.197	0.200	6748.2	6543.5	6697.0	6662.9	106.5
L-ascobic acid		25	0.205	0.199	0.189	6952.9	6645.9	6134.1	6577.6	413.7
		50	0.128	0.125	0.121	3012.3	2858.8	2654.0	2841.7	179.7
		100	0.089	0.092	0.095	1016.4	1169.9	1323.4	1169.9	153.5
control	H_2O_2 2 mM	0	0.146	0.141	0.135	3933.5	3677.6	3370.5	3660.5	281.9
	no H_2O_2 2 mM	0	0.273	0.286	0.280	10433.0	11098.3	10791.2	10774.1	333.0

Note: Each well was originally seeded with 10,000 fibroblast cells

Figure 19 Histogram comparing the number of viable cells following incubation of fibroblasts with mixture of 2 mM H_2O_2 and different antioxidants at 0 (control), 25, 50 and 100 μg/ml. The number of cells without H_2O_2 and antioxidant co-treatment was also provided for comparison (untreated group) (Data = mean ± SD, n = 3)



Note: Each well was originally seeded with 10,000 fibroblast cells

* $P < 0.05$, Dunnett's test (greater than control or having protective effect)

** $P < 0.05$, Dunnett's test (less than control or having cytotoxic effect)

Oxyresveratrol gave the number of viable cells higher than the control (3660.5 ± 281.9 cells), with the mean values of 7345.3 ± 179.7, 6424.1 ± 128.8 and 7908.2 ± 384.1 cells at 25, 50 and 100 μg/mL, respectively, or equivalent to 2-, 1.8- and 2.2-fold increase over control. Puag-Haad also gave protective effect profile similar to oxyresveratrol. At 25, 50 and 100 μg/mL, the number of viable cells increased to 6901.7 ± 204.7, 6748.2 ± 204.7 and 7396.5 ± 128.8 cells or about 1.9-, 1.8- and 2- fold increase over control, respectively.

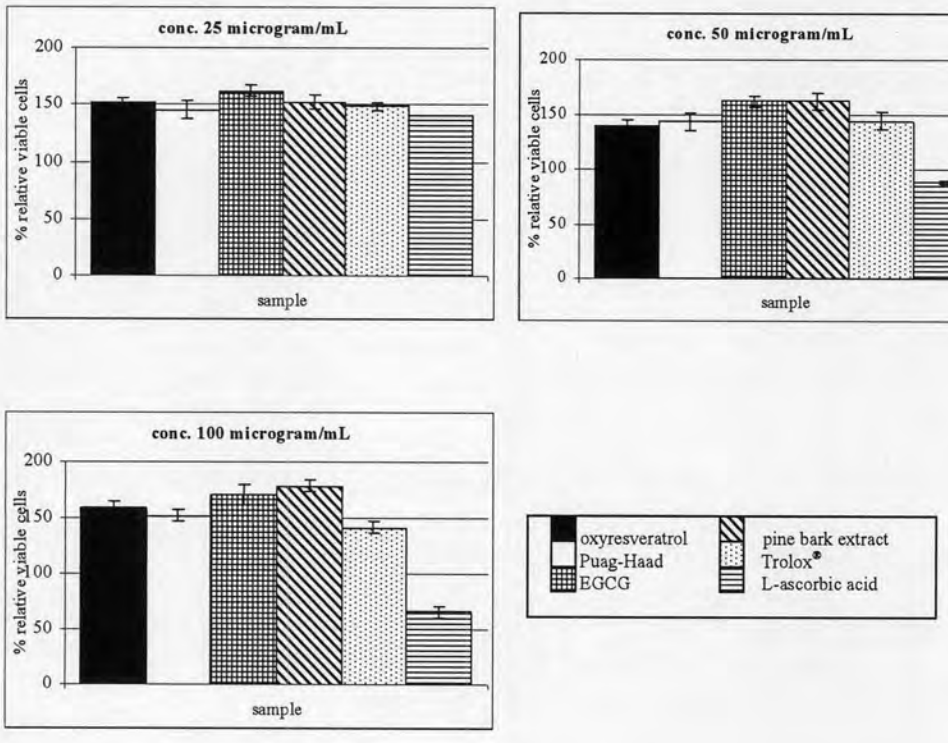
From the data in Table 10, EGCG showed a respective increase in the number of viable fibroblasts by 2.2, 2.2 and 2.4 folds over control at 25, 50 and 100 μg/mL. The French pine bark extract appeared to increase the number of viable cells by 2, 2.2 and 2.5 folds over control whereas Trolox® increased by 2, 1.9 and 1.8 folds at the same concentrations. Increasing the antioxidant concentration from 25 to 100 μg/mL did not seem to further enhance the protective effect of the above antioxidants, as the number of viable cells did not increase proportionately with concentration. The values remained relatively the same or only slightly increased for Puag-Haad, oxyresveratrol, EGCG, Trolox® and pine bark extract.

On the contrary, L-ascorbic acid demonstrated a protective effect only at 25 μg/mL concentration, equivalent to 1.8 folds over control, whereas at concentrations of 50 and 100 μg/mL, the number of surviving fibroblasts drastically dropped to below that of control by about 1.3 and 3.1 folds. This suggested that L-ascorbic acid might potentiate the toxicity of H_2O_2 at higher concentration. The reason for this observation was unclear. However, it is known that L-ascorbic acid is involved in the regeneration of Fe^{2+}, an ion that plays a key role in the formation of highly toxic hydroxyl radical in the presence of H_2O_2 (Halliwell et al., 1992; Cadenas, 1995). It is possible that some trace of irons may exist in the medium, especially from the fetal bovine serum, and this may have resulted in the additional oxidative damages. Recently, it has been demonstrated that L-ascorbic acid could not scavenge singlet oxygen and thus failed to protect red blood cells against UV-induced hemolysis *in vitro* whereas the damage was significantly reduced in the presence of Puag-Haad, oxyresveratrol and pine bark extract (Wachiranuntasin, 2006). The acidity of L-ascorbic acid at high concentration might also be another factor contributing to the observed results. The negative behavior of L-ascorbic acid had been observed in the previous experiment in which a cytotoxic effect was detected at 250 μg/mL concentration using MTT assay.

It should also be noted that none of the antioxidants studied had a complete protection against H_2O_2–induced cytotoxicity as the number of viable cells was still visually smaller than the untreated group in all cases (Figure 20). In addition, the mechanisms by which these antioxidants exerted the protective effect are still unknown and require further investigation. Changes in the experimental conditions such as concentration of H_2O_2, time of exposure and the order of treatment

(pretreatment with antioxidant instead of co-treatment) may result in different protection profiles. The concentration of H_2O_2 and exposure time used in this study (2 mM for 2 hr) was modified from Okano et al. (2003), who reported that incubation of fibroblast with 2 mM of H_2O_2 for 2 hr in Hank's buffered solution (HBS) resulted in 75 % cell loss. In the current study, the stress condition gave the average number of surviving fibroblasts of 3660.5 cells, about 66 % drop from the untreated cells (10774.1 cells). This condition was thus sufficient to produce substantial cell loss, which facilitated detection of any protective effect exerted by the test antioxidants.

Figure 20 Histograms comparing percentage of viable fibroblasts relative to control (100 %) after exposure to 2 mM H_2O_2 with and without antioxidants (Data = mean $\pm$ SD, n = 3)



2.2 MTT assay of ultraviolet –induced cell damages and effect of antioxidants

The spectrum of ultraviolet light ranges from 100 to 400 nm and is divided into UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (<280 nm). UV-A is the predominant genotoxic exposure in the natural environment. It causes indirect genotoxic and cytotoxic effects via sensitizers (Berneburg et al., 2000). The absorbed energy is transferred to oxygen and leads to formation of reactive oxygen species (ROS), which in turn react with various cellular components such as lipids, carbohydrates and proteins. ROS are also responsible for a number of mutagenic lesions to the DNA. 8-Oxoguanine (8-oxo-G), which mispairs with adenine and induces GC→AT transition, is the most common DNA alteration. Besides 8-oxo-G numerous other damages are known to be induced by UV-A radiation, such as thymidine glycol and single strand breaks (Berneburg et al., 2000). UV-A is also known to penetrate the skin more deeply than UV-B and can easily reach the dermis layer of the skin (Berneburg et al., 2000; Scharffetter-Kochanek et. al., 2000). Thus, it is highly relevant to evaluate the effect of UV-A using fibroblasts as the model since they are majority of the dermal cells.

The protective effect of Puag-Haad and reference antioxidants against UV-A-induced cell damage was evaluated by exposure of the fibroblasts to 20 J/cm^2 of UVA following overnight treatment with the test antioxidants. MTT test was used to determine the number of surviving fibroblasts in each group.

Similar to previous MTT test, the data in Table 11 and Figure 21 demonstrated that the standard curve was linear in the range of 0 to 20,000 cells with the coefficient of determination (R^2) of 0.9710 (Table 12), indicating the validity of the MTT test for this study.

Table 11 Data of standard curve between absorbance (OD) and cell number

Cell number (x)	OD1	OD2	OD3	average OD (y)
0	0.140	0.138	0.131	0.136
500	0.137	0.143	0.148	0.143
1000	0.164	0.150	0.156	0.157
7000	0.226	0.258	0.283	0.256
15000	0.407	0.385	0.378	0.390
20000	0.426	0.424	0.395	0.415

Figure 21 Standard curve from the relationship between OD and cell number

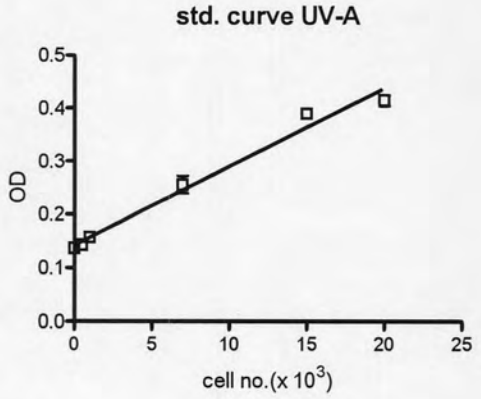


Table 12 Data were interpolated from linear regression

Best-fit values	
Slope	0.00001478 ± 0.0000006387
Y-intercept	0.1422 ± 0.006776
X-intercept	-9622
1/slope	67660
95% Confidence Intervals	
Slope	0.00001343 to 0.00001613
Y-intercept when X=0.0	0.1279 to 0.1566
X-intercept when Y=0.0	-11750 to -7777
Goodness of Fit	
R^2	0.971
Sy.x	0.02099
Is slope significantly non-zero?	
F	535.5
DFn, DFd	1.000, 16.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	6
Maximum number of Y replicates	3
Total number of values	18
Number of missing values	0

Equation $y = 0.00001478x + 0.1422$

$r^2 = 0.9710$

Table 13 shows the absorbance and number of viable fibroblasts remaining after UV-A irradiation in the presence of Puag-Haad at 25, 50 and 100 μg/mL using the MTT assay. The results were also compared to cells exposed to other antioxidants (oxyresveratrol, EGCG, French pine bark extract, L-ascorbic acid or Trolox®) at the same antioxidant concentrations and UV-A dose as shown in Figure 22. Histograms comparing percentage of viable fibroblasts relative to control (100 %) are also shown in Figure 23. It was found that all the antioxidants were able to provide protective effect against UV-A in a concentration-dependent manner. As the concentration of each antioxidant was increased from 25 to 100 μg/mL, the number of viable fibroblasts increased accordingly and became higher than control, which was exposed to only UV-A (no antioxidant present). The untreated group, which was not exposed to any UV radiation nor any antioxidant, showed the highest number of viable fibroblasts with the mean value of 27907.1 $\pm$ 1257.3 cells whereas the UV-irradiated control group gave the average cell number of 7767.3 $\pm$ 473.6 cells, indicating that this dose of UV-A caused about 3.6 fold loss in cell viability.

Data on the number of viable cells exposed to UV-A, with and without antioxidants, were then analyzed by one-way ANOVA at 5% level. Significant differences were found among the six antioxidants and the control group ($P < 0.05$). Consequently, post-ANOVA Dunnett's test was further applied. The number of surviving cells increased and became significantly greater than the control for all antioxidants ($P < 0.05$; Dunnett's test), particularly at 100 μg/mL, indicating the individual antioxidant's ability to protect the cells from UV-A-induced damages. The average number of viable fibroblasts at this concentration, in a decreasing order, was 27839.4 (L-ascorbic acid), 16968.9 (Trolox©), 13653.6 (Pine bark extract), 13292.7 (oxyresveratrol), 12977.0 (Puag-Haad) and 12232.7 (EGCG), which was equivalent to 3.6, 2.2, 1.8, 1.7, 1.7 and 1.6 fold increase over control, respectively. At lower concentration of 50 μg/mL, the protective effect decreased for all antioxidants but the number of surviving cells was still significantly higher than the control in the case of

oxyresveratrol and L-ascorbic acid, with the respective mean values of 11353.2 and 23712.2 cells or equivalent to 1.5- and 3.1- folds over control ($P < 0.05$; Dunnett's test). However, the values were not different from control ($P > 0.05$) for EGCG, Puag-Haad, Trolox© and pine bark extract, of which the average cell number was in the range of 5737.5 – 9210.6 cells or about 0.7 – 1.2 folds over control. At 25 μg/mL, the protective effect disappeared for all antioxidants except L-ascorbic acid, which still exhibited significantly greater number of viable cells than the control with the mean value of 14871.4 cells or about 1.9 fold increase over control ($P < 0.05$; Dunnett's test). The other antioxidants (Puag-Haad, oxyresveratrol, EGCG, pine bark extract and Trolox©), on the contrary, yielded the values of viable cells which were significantly lower than the control group ($P < 0.05$; Dunnett's test), indicating that this concentration was too low to provide protection against UV-A and could potentiate its cytotoxicity by some unknown mechanisms.

Thus, the results from the current study showed that all the antioxidants tested, including Puag-Haad and oxyresveratrol, could provide protection against UV-A induced cytotoxicity. However, Puag-Haad, oxyresveratrol, EGCG, pine bark extract and Trolox© appeared to provide protection only at high concentration (100 μg/mL), whereas L-ascorbic acid was the most effective and could provide protection at all three concentrations studied.

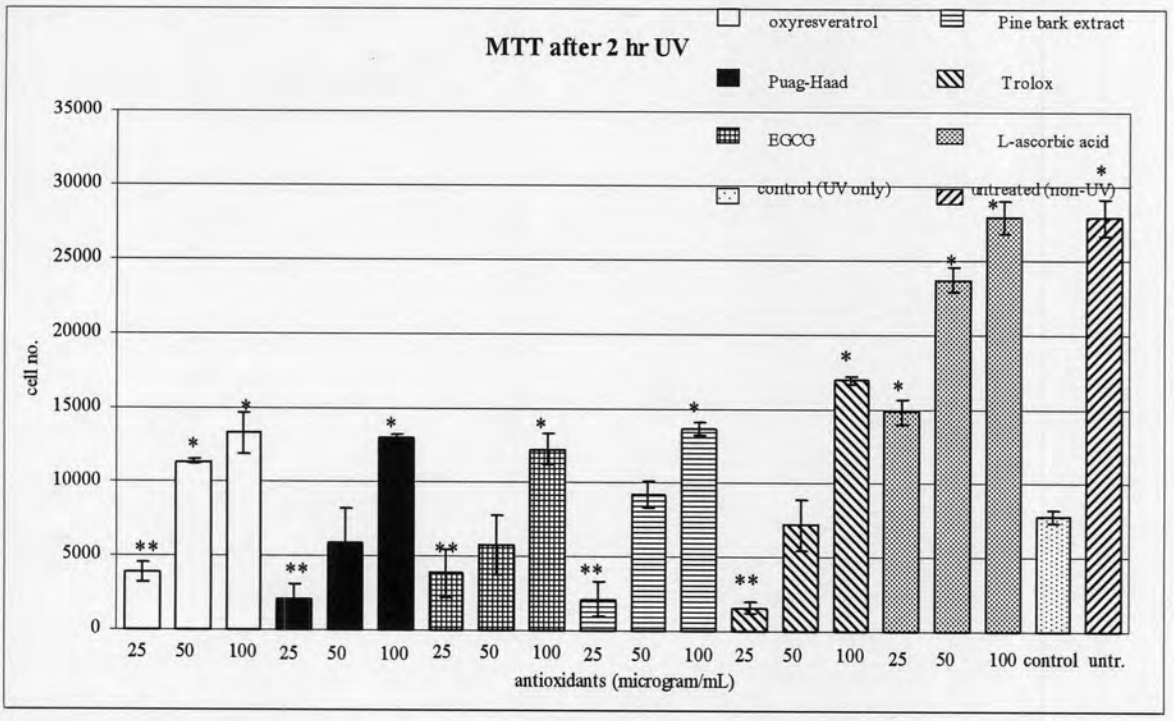
The reasons to explain the observed results are still not known at present. L-ascorbic acid appeared to be the least effective against H_2O_2-induced damage in the previous study. Using the same concentration range (25 - 100 μg/mL), L-ascorbic acid could provide protection only at 25 μg/mL but seemed to potentiate the toxic effect of H_2O_2 at 50 and 100 μg/mL. It is likely that the optimum concentration for L-ascorbic acid and other antioxidants in protecting (or potentiating) cell damages might be different when using different oxidative stresses and experimental conditions. More studies using larger concentration range therefore should be conducted to support this hypothesis. L-ascorbic acid might also directly react with H_2O_2 and result in concentration-dependent degradative products that could be more toxic to the fibroblasts (Halliwell et al., 1997; Minetti, 1992; Fisher and Naughton, 2004).

Table 13 OD values and number of viable fibroblasts remaining after UV-A irradiation in the presence of different antioxidants using MTT assay.

sample		Conc. (µg/mL)	OD1	OD2	OD3	cell no.1	cell no. 2	cell no.3	Average	S.D.
Oxyresveratrol	UV 2 hr	25	0.210	0.189	0.200	4587.3	3166.4	3910.7	3888.1	710.7
		50	0.308	0.309	0.313	11217.9	11285.5	11556.2	11353.2	179.0
		100	0.359	0.318	0.339	14668.5	11894.5	13315.3	13292.7	1387.1
Puag-Haad		25	0.165	0.191	0.162	1542.6	3301.8	1339.6	2061.3	1079.0
		50	0.223	0.267	0.197	5466.8	8443.8	3707.7	5872.8	2394.0
		100	0.331	0.339	0.332	12774.0	13315.3	12841.7	12977.0	294.9
EGCG		25	0.219	0.205	0.173	5196.2	4249.0	2083.9	3843.0	1595.4
		50	0.226	0.198	0.257	5669.8	3775.4	7767.3	5737.5	1996.8
		100	0.307	0.338	0.324	11150.2	13247.6	12300.4	12232.7	1050.4
Pine bark		25	0.174	0.156	0.191	2151.6	933.7	3301.8	2129.0	1184.2
		50	0.293	0.271	0.271	10203.0	8714.5	8714.5	9210.6	859.4
		100	0.341	0.339	0.352	13450.6	13315.3	14194.9	13653.6	473.6
Trolox		25	0.160	0.172	0.165	1204.3	2016.2	1542.6	1587.7	407.8
		50	0.274	0.249	0.223	8917.5	7226.0	5466.8	7203.4	1725.4
		100	0.397	0.393	0.389	17239.5	16968.9	16698.2	16968.9	270.6
L-ascobic acid		25	0.375	0.360	0.351	15751.0	14736.1	14127.2	14871.4	820.3
		50	0.480	0.505	0.493	22855.2	24546.7	23734.8	23712.2	846.0
		100	0.572	0.540	0.549	29079.8	26914.7	27523.7	27839.4	1116.5
control	UV 2 hr	0	0.250	0.264	0.257	7293.6	8240.9	7767.3	7767.3	473.6
	no UV 2 hr	0	0.542	0.546	0.576	27050.1	27320.7	29350.5	27907.1	1257.3

Note: Each well was originally seeded with 10,000 fibroblast cells

Figure 23 Histogram comparing the number of viable cells following irradiation of fibroblasts with UV-A (20 J/cm^2) in the presence of different antioxidants at 0 (control), 25, 50 and 100 μg/ml. The number of cells without UV-And antioxidant was also provided for comparison (untreated group) (Data = mean ± SD, n = 3)

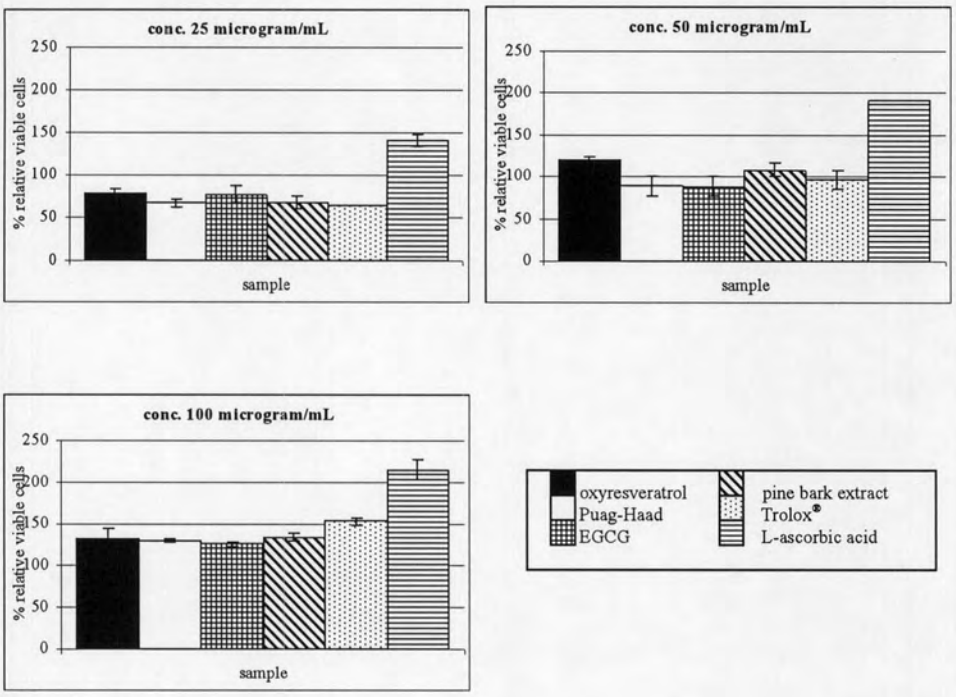


Note: Each well was originally seeded with 10,000 fibroblast cells

* P < 0.05, Dunnett's test (greater than control or having protective effect)

** P < 0.05, Dunnett's test (less than control or having cytotoxic effect)

Figure 22 Histograms comparing percentage of viable fibroblasts relative to control (100%) after UV-A irradiation with and without antioxidants
(Data = mean ± SD, n = 3)



2.3 Effect of antioxidants on UV-A induced LDH leakage

To further evaluate the deleterious effect of UV-A and its possible mechanisms on fibroblasts, the extent of lactate dehydrogenase (LDH) enzyme was also determined.

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme which is not secreted outside the cells. But upon damage of the cell membrane LDH leaks out. Measurement of the release of LDH from cells is based on a colorimetric quantitation after an enzymatic reaction (Vihola et al., 2005). The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of tetrazolium dye. LDH leakage was measured from normal human dermal fibroblast cells using TOX-7 cytotoxicity assay-kit. The % release of Puag-Haad and other antioxidants were compared at various concentrations (25, 50 and 100 μg/mL). As previously described in the method, the extent of % release was calculated by comparison of the individual absorbance with the average absorbance of the total LDH released from the lysed cells.

Table 14 and Figure 24 show the average values of percent LDH release from normal human dermal fibroblasts following 24 hr-incubation with Puag-Haad, oxyresveratrol, EGCG, pine bark, Trolox® or L-ascorbic acid before exposure with UV-A 20 J/cm^2. The raw data are also given in Appendix F. As expected, exposure of fibroblasts to UV-A alone without pretreatment with antioxidants induced some release of LDH with the mean value of 19.10 $\pm$ 1.33 %. This was probably due to the action of ROS (induced by UV-A irradiation) on the cell membrane components which led to perturbation of membrane integrity and leakage of LDH. Several studies have been reported that ROS caused mitochondrial dysfunction by disturbing the equilibrium of proteins and loss of the mitochondrial transmembrane potential (Ouyang et al., 2004; Su et al., 2005; Lee et al., 2005) and this could be due to the no FBS in fibroblasts culture, so cells were easy susceptible to oxidative stress e.g. UV-A or antioxidants. Addition of antioxidants therefore should be able to revert this damaging effect by scavenging the ROS. Puag-Haad, oxyresveratrol, EGCG, pine bark extract, L-ascorbic acid and Trolox© had previously been demonstrated to scavenge ROS like DPPH, superoxide anion radical and hydroxyl radical (Wachiranuntasin, 2005). However, pretreatment of the cells with these antioxidants

followed by UV-A irradiation resulted in a concentration-dependent increase of LDH release instead of reducing the LDH leakage. This observation suggested a direct irritating effect of the individual antioxidant on the cell membrane and this effect was apparently greater than their ROS-scavenging activity. Therefore, the overall result was a further increase in the LDH release from the control.

Further experiments by exposing fibroblasts to Puag-Haad or EGCG alone without UV-A exposure confirmed that the antioxidants could induce a concentration-dependent release of LDH by themselves as shown in Table 15. The fibroblasts without UV-A or antioxidant pretreatment did not give any LDH release. However, when the cells were incubated overnight (24-hr) with 25 and 100 μg/mL Puag-Haad, LDH release increased to 0.36 ± 0.16 and 24.77 ± 0.69 %, respectively. Similar result was also observed for EGCG. The data thus agreed with the cytotoxicity test based on MTT measurements (part 1.2) in that the cytoxic effect of the test antioxidants was concentration-dependent and may not relate to their ROS-scavenging activity or their protective effect against ROS- induced damages.

Also, it is interesting to note that Puag-Haad gave % LDH release lower than oxyresveratrol, especially at 25 and 50 μg/mL (Table 14). At 25 μg/mL the extents of LDH release were 37.73 ± 0.97 % and 58.75 ± 1.00 % for Puag-Haad and oxyresveratrol, respectively. As the exposure concentration was increased to 50 μg/mL, the extents of LDH release increased to 45.16 ± 4.13 % % for Puag-Haad and to 61.41 ± 0.63 % for oxyresveratrol. The smaller extent of LDH release suggested that Puag-Haad induced less membrane irritation than oxyresveratrol in this concentration range.

Data in Table 14 and Figure 24 also show that at 25 μg/mL concentration, the membrane-irritating effect of oxyresveratrol under UV-A influence was similar to EGCG and L-ascorbic acid, with the average LDH release of 58.75, 63.80 and 56.64 %, respectively. Trolox®, pine bark extract and Puag-Haad, on the other hand, were somewhat less irritating, giving an average % LDH release in the same range (33.69 to 37.73%). At 50 μg/mL concentration, the LDH release slightly increased and appeared to level off for all antioxidants. At 100 μg/mL, L-ascorbic acid gave the highest LDH release under UV-A (77.34%), followed by EGCG (71.60%). Oxyresveratrol, pine bark extract, Trolox© and Puag-Haad appeared to produce similar

LDH release, with the respective mean values of 61.92, 61.01, 60.14 and 57.69%. Thus, although the results from part 2.2 (MTT assay on UV-A irradiated fibroblasts) suggested the ability of all test antioxidants to protect the cells against UV-A through their ROS scavenging activity, they also were capable of directly interacting with the fibroblast cell membrane, which caused the release of LDH into the medium.

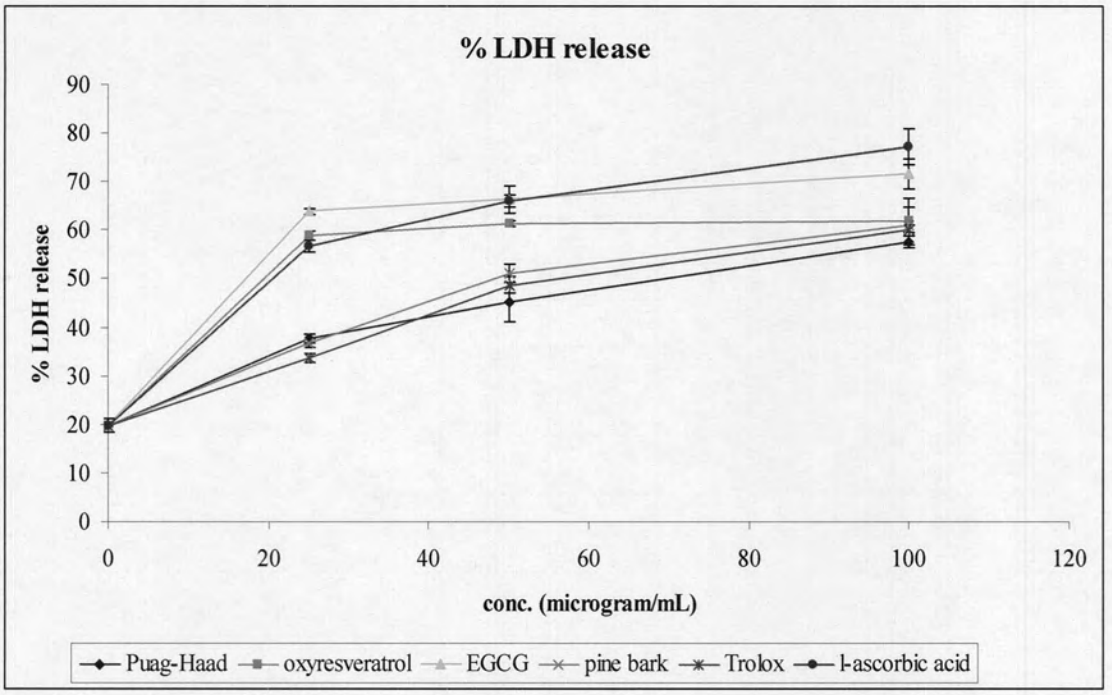
Table 14 Percent LDH release from normal human fibroblasts following exposure to UV-A (20 J/cm^2) with and without antioxidant pretreatment (Mean $\pm$ SD, n = 3).

Conc. (μg/mL)	Puag-Haad		oxyresveratrol		EGCG		Pine bark		L-ascorbic acid		Trolox®	
	%LDH released	SD	%LDH released	SD	%LDH released	SD	%LDH released	SD	%LDH released	SD	%LDH released	SD
0	19.70	1.33	19.70	1.33	19.70	1.33	19.70	1.33	19.70	1.33	19.70	1.33
25	37.73	0.97	58.75	0.10	63.80	0.59	36.74	1.04	56.64	1.30	33.69	1.03
50	45.16	4.13	61.41	0.63	66.38	2.91	50.94	1.85	66.11	1.25	48.67	1.65
100	57.69	1.19	61.92	4.84	71.60	3.15	61.01	3.92	77.34	3.78	60.14	0.78

Table 15 Percent LDH release from normal human fibroblasts following exposure to Puag-Haad and EGCG without UV-A (Mean $\pm$ SD, n = 3).

Conc. (μg/mL)	Puag-Haad		EGCG	
	%LDH released	SD	%LDH released	SD
0	0.00	0.00	0.00	0.00
25	0.36	0.16	0.64	0.16
100	24.77	0.69	9.84	0.55

Figure 24 Plot % LDH release from fibroblasts following exposure to UV-A with and without antioxidant pretreatment. (Data = mean ± SD, n = 3).



Part 3. Effect of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and its active constituent (oxyresveratrol) on the reduction of DNA damage in cells exposed to UV-A

The purpose of this part was to evaluate whether Puag-Haad and oxyresveratrol could have a protective effect on the DNA damage and thus could reduce the extent of cell death following UV-A exposure of cultured fibroblasts. The extent of cell death, either apoptosis or necrosis, can be quantitated by assessing the state and content of nuclear DNA through a flow cytometric analysis of red fluorescence from fixed propidium iodide-stained, RNase-treated cells.

Propidium iodide (PI) is a well known dye used for the detection of late apoptosis process (Lecoeur, 2002). It can stain DNA by intercalating between the bases with a stoichiometry of one dye per 4-5 base pairs of DNA. It was used in this study to clarify the DNA content in the fibroblast cells after exposure to oxidative stress such as UV-A in the presence of Puag-Haad or oxyresveratrol.

Cell division cycle consists of two major stages: interphase (consisting of G_1, S and G_2 phases) and M phase (consisting of mitosis and cytokinesis). In addition, cells that have temporarily or reversibly stopped dividing (as in mature or fully differentiated cells), are said to have entered a state of quiescence called G_0 phase. On the other hand, cells that have permanently stopped dividing due to age or accumulated DNA damage are said to be senescent. Cellular senescence is a state that occurs in response to DNA damage or degradation that makes a cell's progeny non-viable.

Flow cytometry has been used to analyze numerous features of cell death such as apoptosis (Lecoeur, 2002). PI fluorescence representation of apoptotic and other non-viable cells is heralded by subG_0/G_1 events on DNA histograms (Nicoletti et al. 1991), from which special algorithms are used for DNA content analysis. The area under the curve of the plot between the cell count and fluorescence intensity is integrated. Then, the percentages of population in each phase of cell cycle are calculated. Thus, a more complete analysis of the apoptotic progression may be revealed by two-parameter flow cytometric analysis than would be evident if the DNA content was the only feature monitored (Worrington et al., 2003).

Data were analyzed by bivariate analysis of log10 forward-angle light scatter (FALS) or DNA signals from a population of cells, as represented by PI fluorescence on the X-axis of the DNA histograms. After staining by PI, DNA of viable, dividing cells give fluorescence in the region of G_0/G_1 phase (the transition between quiescence and G_1 phases) through the S phase, during which DNA is synthesized and doubled in amount from diploid (G_1 phase) to tetraploid (G_2 phase), as well as in the M phase, after which daughter cells are produced. Cells that undergo cellular senescence or cell apoptosis will become non-viable and have low DNA content, thereby producing very low fluorescence intensity after PI staining. This will give rise to an early peak in the low intensity region of the DNA histogram. The percentage of cells in this region ($subG_0/G_1$ phase) can be used as a parameter of the extent of DNA damage or cell death (BD biosciences, 2003).

The percentages of fibroblasts in $subG_0/G_1$ phase by flow cytometry are shown in Table 16. The $subG_0/G_1$ peak in flow cytometric detection is considered as an indicator of cell apoptosis or non-viable cells. Figures 25A–F are representatives of plots between cell counts and fluorescence intensity showing the number of fibroblasts in various phases of cell division cycle as well as in the non-viable phase (the $subG_0/G_1$ phase) taken from the untreated cells (A), UV-A-treated cells (B), UV-A plus pretreatment with Puag-Haad (C,D) or with oxyresveratrol (E,F), respectively.

The results showed that the percentage of non-viable cells ($subG_0/G_1$ peak) increased from 55.85 $\pm$ 2.47 % in untreated cells (no UV-A) to 88.33 $\pm$ 12.05 % in cells exposed to UV-A 20 J/cm^2 without any added antioxidants (Table 16). This suggested that most of the fibroblasts treated with UV-A became non-viable with low DNA content were weakly stained by PI giving low fluorescence intensity. The percentage of non-viable cells in the $subG_0/G_1$ peak slightly decreased to 78.94 $\pm$ 12.49 % when the cells were incubated for 24 hr with 25 µg/mL Puag-Haad prior to UV-A exposure and further reduced to 73.85 $\pm$ 9.61 % with 50 µg/mL Puag-Haad. Similarly, incubation of fibroblasts with 25 and 50 µg/mL oxyresveratrol before UV-A exposure could decrease the percentage of non-viable cells to 75.65 $\pm$ 9.36 % and 73.92 $\pm$ 10.19 %, respectively. Also, it is interesting to note that the number of non-viable cells ($subG_0/G_1$ phase) of the untreated fibroblasts was already high without any UV-exposure (55.85 $\pm$ 2.47 %). This could be due to the apoptotic body interfere at

the same intensity. However, the staining conditions and time before flow cytometric analysis were the same for all groups, which should still allow valid comparison.

UV radiation is known to cause DNA damages via generation of harmful ROS leading to premature cell death (Berneburg et al., 2000). Thus, it is not surprising to see the number of non-viable fibroblasts in the subG_0/G_1 region to rise sharply following UV-A exposure (Figure 25B vs 25A), which is one of the most penetrating type of UV radiation being able to reach the dermis (Berneburg, Plettenberg and Krutmann, 2000; Scharffetter-Kochanek et. al., 2000). Comparison of Figure 25A and 25B also shows that apart from the rise in the non-viable cells (subG_0/G_1 peak), there is a concomitant decrease in the number of viable cells especially in the G_0/G_1 region after UV exposure as opposed to the untreated fibroblasts (no UV). As seen in Table 17, the percentage of viable cells in the G_0/G_1 region of the untreated fibroblasts was 26.06 ± 3.28 %, whereas the extent decreased to 3.34 ± 1.23 % in the control group after UV-A exposure. Pretreatment with Puag-Haad or oxyresveratrol also was able to partially reverse this effect. For example, in fibroblasts incubated for 24 hr with 25 and 50 μg/mL Puag-Haad followed by UV-A irradiation, the percentage of viable cells in the G_0/G_1 region increased to 15.21 ± 9.37 % and 17.81 ± 7.93%, respectively. Likewise, pretreatment with 25 and 50 μg/mL oxyresveratrol increased the number from control to 17.76 ± 7.09 % and 19.96 ± 6.51%, respectively.

The percentages of cells in the subG_0/G_1 and G_0/G_1 regions were then statistically analyzed comparing the control with Puag-Haad or oxyresveratrol using ANOVA at 5% significance level. No significant differences were found in the subG_0/G_1 values between the control and Puag-Haad or between the control and oxyresveratrol at both concentrations ($P > 0.05$). However, when the values in the G_0/G_1 region was analyzed, significance was found ($P < 0.05$) for oxyresveratrol and further Dunnett's test revealed that both 25 and 50 μg/mL concentrations of oxyresveratrol were able to significantly increase the number of viable cells from the control group ($P < 0.05$). Although pretreatment with Puag-Haad did not result in significant increase of the cells in the G_0/G_1 region, the extent of increase was similar to oxyresveratrol (Table 17). Histograms comparing different treatment groups are also shown in Figures 26 and 27 for the subG_0/G_1 phase and G_0/G_1 phase, respectively.

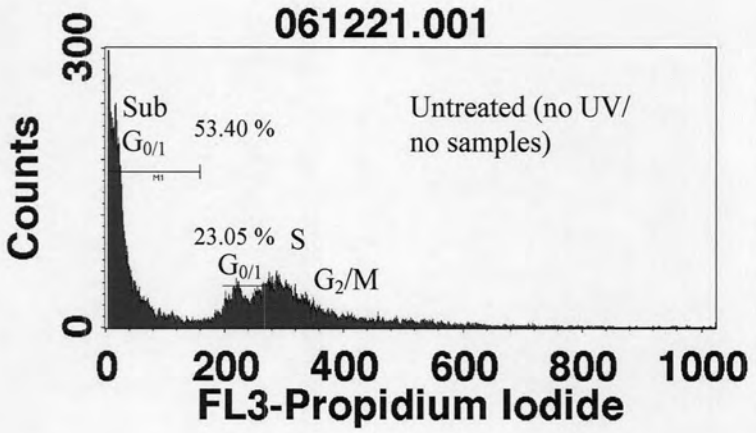
Table 16. The percentage of fibroblasts in subG_0/G_1 phase by flow cytometry with propidium iodide staining following exposure to UV-A with and without antioxidant pretreatment

Sample	% sub G0/G1 phase (n1)	% sub G0/G1 phase (n2)	% sub G0/G1 phase (n3)	Average	SD
Untreated	53.40	55.81	58.34	55.85	2.47
Control	96.99	93.43	74.57	88.33	12.05
Puag-Haad 25µg/mL	86.73	85.56	64.53	78.94	12.49
Puag-Haad 50µg/mL	79.29	79.50	62.75	73.85	9.61
oxyresveratrol 25µg/mL	79.68	82.33	64.95	75.65	9.36
oxyresveratrol 50µg/mL	78.26	81.22	62.28	73.92	10.19

Table 17. The percentage of fibroblasts in G_0/G_1 phase by flow cytometry with propidium iodide staining following exposure to UV-A with and without antioxidant pretreatment

Sample	% G0/G1 phase (n1)	% G0/G1 phase (n2)	% G0/G1 phase (n3)	Average	SD
Untreated	23.05	25.57	29.56	26.06	3.28
Control	1.97	3.71	4.34	3.34	1.23
Puag-Haad 25µg/mL	10.51	9.11	26.00	15.21	9.37
Puag-Haad 50µg/mL	13.39	13.08	26.96	17.81	7.93
oxyresveratrol 25µg/mL	14.29	13.07	25.92	17.76	7.09
oxyresveratrol 50µg/mL	17.66	14.91	27.31	19.96	6.51

(A)



(B)

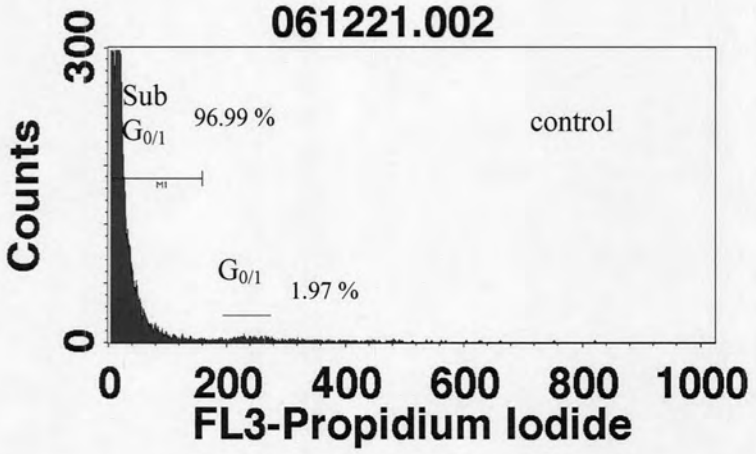
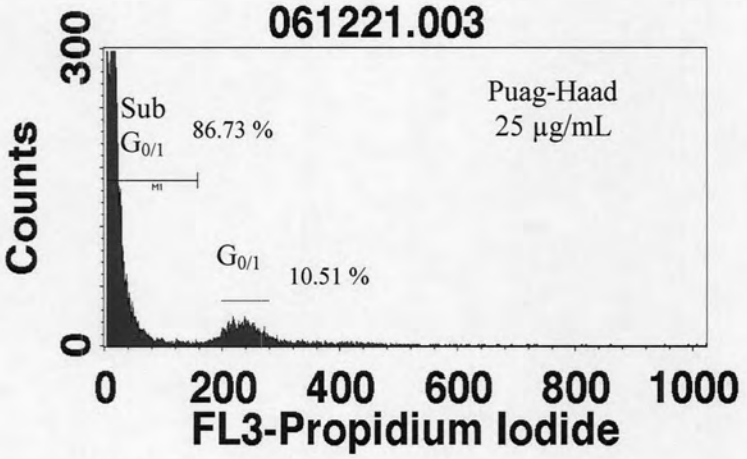


Figure 25. Representative DNA histograms after flow cytometric analysis showing the effect of UV-A irradiation, with and without antioxidant pretreatment, on the amount of fibroblasts in the non-viable state (subG_0/G_1) and in various phases of cell division cycle (G_0/G_1, S, G_2/M phases). (A) = untreated cells (no UV-A); (B) = control (UV-A only). 15,000 cells counted per run.

(C)



(D)

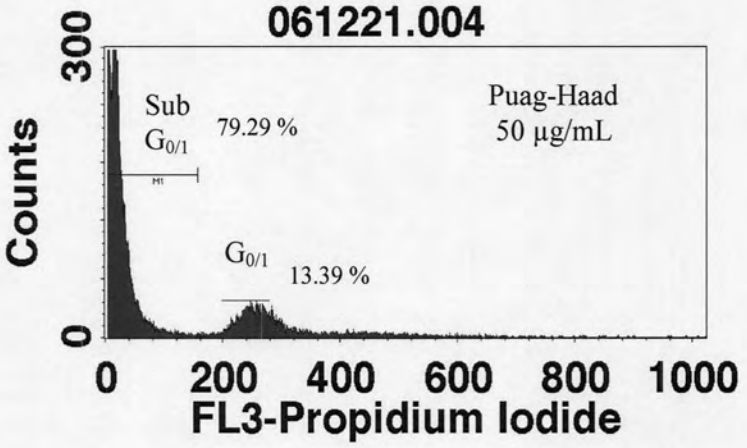
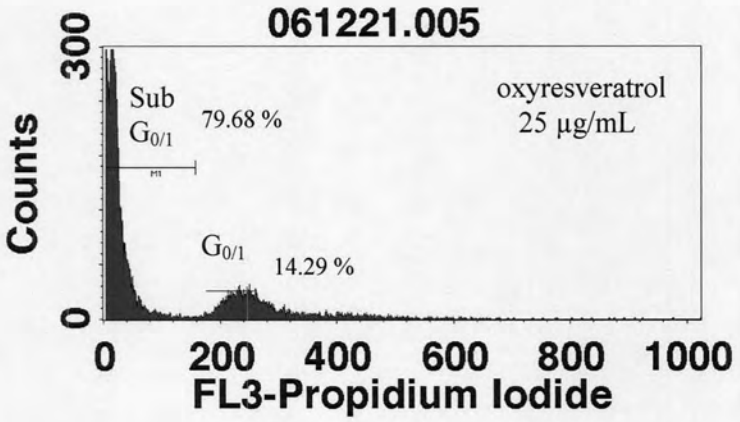


Figure 25 (cont.). Representative DNA histograms after flow cytometric analysis showing the effect of UV-A irradiation, with and without antioxidant pretreatment, on the amount of fibroblasts in the non-viable state (subG_0/G_1) and in various phases of cell division cycle (G_0/G_1, S, G_2/M phases). (C) = UV-A with 25 µg/mL Puag-Haad; (D) = UV-A with 50 µg/mL Puag-Haad. 15,000 cells counted per run.

(E)



(F)

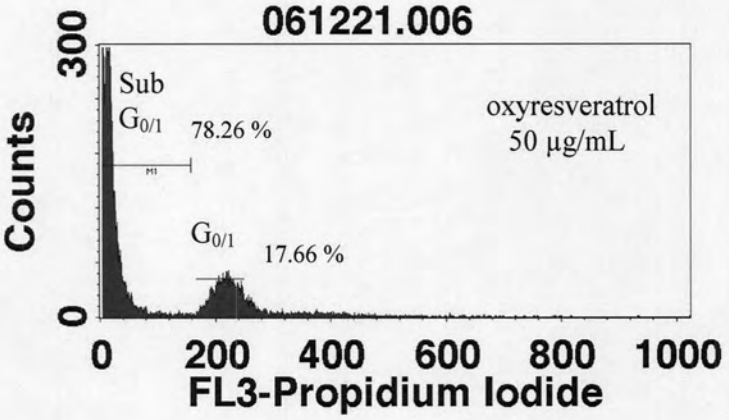
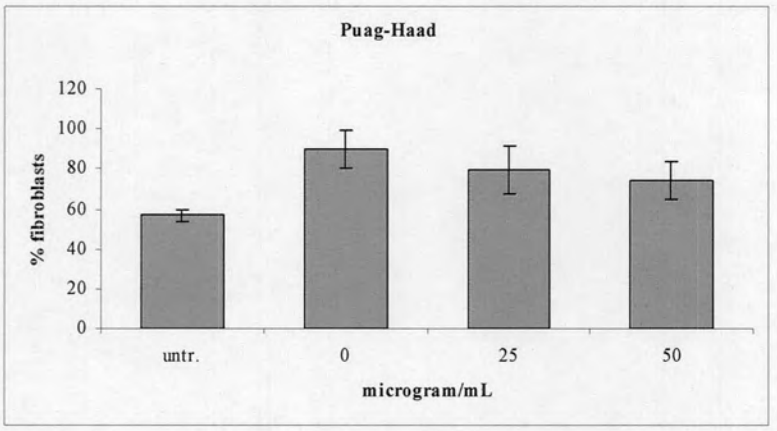


Figure 25 (cont.). Representative DNA histograms after flow cytometric analysis showing the effect of UV-A irradiation, with and without antioxidant pretreatment, on the amount of fibroblasts in the non-viable state (subG_0/G_1) and in various phases of cell division cycle (G_0/G_1, S, G_2/M phases). (E) = UV-A with 25 µg/mL oxyresveratrol; (F) = UV-A with 50 µg/mL oxyresveratrol. 15,000 cells counted per run.

(A)



(B)

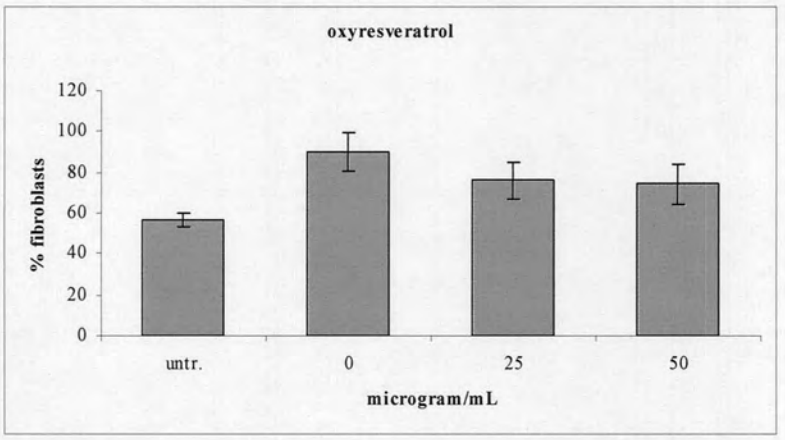
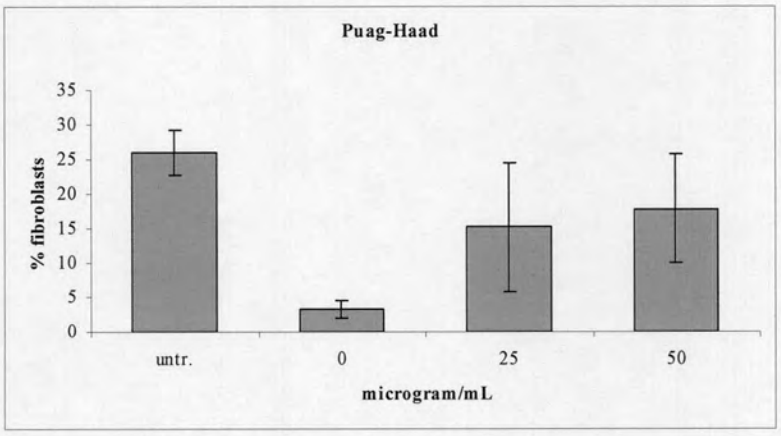


Figure 26. Histograms comparing percentages of fibroblasts in the subG_0/G_1 phase following UV-A irradiation with and without antioxidant pretreatment. (A) = Puag-Haad 25 and 50 μg/mL; (B) = oxyresveratrol 25 and 50 μg/mL. Untr. = untreated cells (no UV, no antioxidant). Data are mean ± SD of three independent experiments.

(A)



(B)

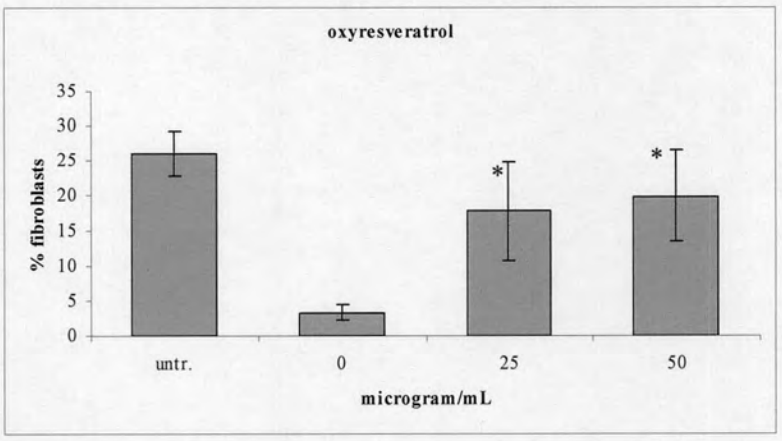


Figure 27. Histograms comparing percentages of fibroblasts in the G_0/G_1 phase following UV-A irradiation with and without antioxidant pretreatment. (A) = Puag-Haad 25 and 50 μg/mL; (B) = oxyresveratrol 25 and 50 μg/mL. Untr. = untreated cells (no UV, no antioxidant). Data are mean $\pm$ SD of three independent experiments. * Significantly greater than control (0 μg/mL) after Dunnett's test ($P < 0.05$)

The present data from flow cytometric analysis agreed with previous MTT results that Puag-Haad and oxyresveratrol were able to prevent or reduce UV-induced cellular damages in the fibroblast model (part 2.2). The two compounds may act probably by scavenging deleterious ROS that can cause damage to the DNA, resulting in prevention or reduction of cell death. In addition to harmful ROS generation, several studies reported that UV radiation could cause mitochondrial dysfunction by disturbing the equilibrium of pro-apoptotic proteins and inducing loss of the mitochondrial transmembrane potential leading to apoptosis (Kimura et al., 1999; Yu-Ying et al., 2004; Sitailo et al., 2002). Therefore, the results from this part indicated that both Puag-Haad and oxyresveratrol could reduce DNA damages caused by UV-A leading to an increase in the number of viable fibroblasts. Their protective effects also appeared to be concentration-dependent.

Part 4. Evaluation of anti-collagenase activity of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and its active constituent (oxyresveratrol)

In the process of wrinkle formation, it was acknowledged that the alterations of collagen amount and character were critical factors. The nature of wrinkling in photo-aging and chronological aging seems to be different. Alterations in the composition of collagen in the dermis may play a role in the phenotypic appearance of aged skin. Light may be functioning as an inducer of certain enzymes, such as collagenase, which may result in damage to collagen molecules. UV-A1 (370-400 nm) may induce collagenase, and gelatinase can be induced by UV-B (300-320 nm) (Qureshi A., 2001).

DQ™ gelatin (fluorescein conjugate gelatin) was used as a substrate which is gelatin that has been labeled by fluorescein to such a degree that the fluorescence is quenched. The substrates typically exhibit less than 3% of the fluorescence of the corresponding free dyes. This substrate is efficiently digested by most gelatinases and collagenases to yield highly fluorescent peptides. Collgenase, Type IV from *Clostridium histolyticum* was used as a control enzyme.

The percentage of anti-collagenase enzyme activity (% inhibition of collagenase) of Puag-Haad compared to those of oxyresveratrol, EGCG, pine bark, L-ascorbic acid, Trolox® at various concentrations (0, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 3.0 and 5.0 mg/mL) are shown in Figure 28.

It was found that as the concentration was increased, the extent of collagenase inhibition also increased for all antioxidants until certain concentrations where the plots reached a plateau and the inhibition became steady (Figure 28 and Table 18). The percent collagenase inhibition of Puag-Haad increased to 99.99 $\pm$ 0.03 % at the concentration of 0.5 mg/mL. This inhibition was higher than those of oxyresveratrol (77.92 $\pm$ 2.76 %), L-ascorbic acid (18.76 $\pm$ 3.15 %) and Trolox® (10.89 $\pm$ 1.56 %) at the same concentration. L-ascorbic acid and Trolox® had the percentage of maximum inhibition of 97.63 $\pm$ 0.1 % and 95.39 $\pm$ 0.14 % at the concentration 2 and 3 mg/mL, respectively.

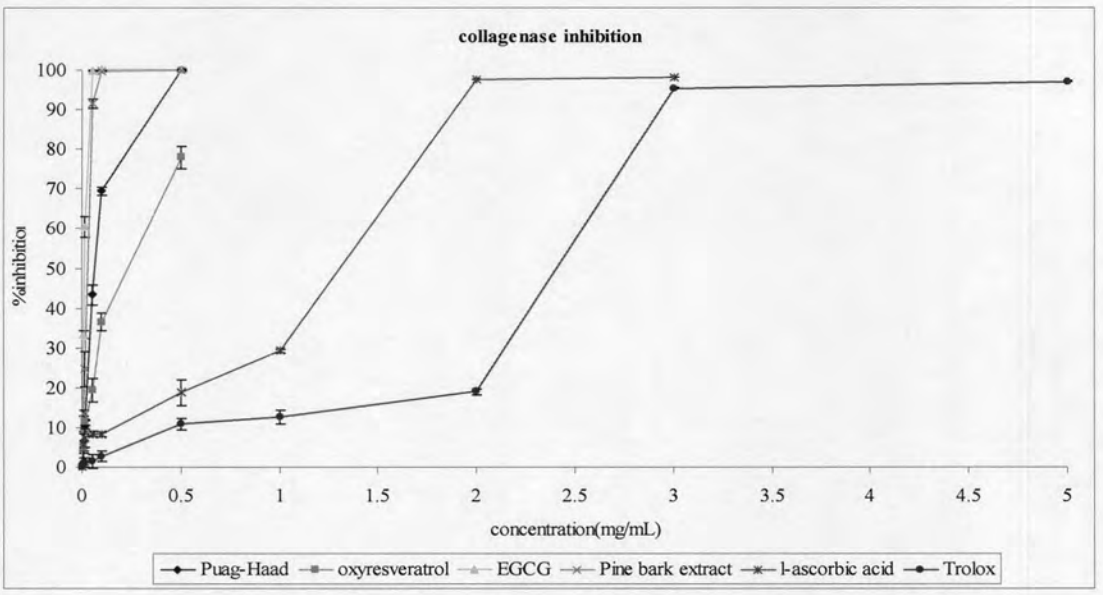
Both EGCG and pine bark demonstrated good collagenase inhibition with respective maximum inhibition of 99.78 $\pm$ 0.13 % and 99.85 $\pm$ 0.09 % observed at

0.05 and 0.1 mg/mL. When the concentration was further increased to 0.5 mg/mL, the inhibition percentage did not increase. Both compounds also had similar inhibition extent of 99.78 – 99.98 % (EGCG) and 91.40 – 99.90 % (pine bark) at the concentration ranging from 0.05 – 0.5 mg/mL (Table 18).

Table 18 Inhibitory effect of Puag-Haad on collagenase compared to other antioxidants at various concentrations (Mean ± SD, n = 3)

No.	Final concentration (mg/mL)	Puag-Haad		oxyresveratrol		EGCG		Pine bark extract		L-ascorbic acid		Trolox®	
		%inh	SD	%inh	SD	%inh	SD	%inh	SD	%inh	SD	%inh	SD
1	0.000	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.005	6.57	2.41	5.04	1.45	33.27	1.03	13.58	0.74	5.52	3.10	0.98	0.27
3	0.010	10.38	1.36	11.33	0.82	60.41	2.73	24.61	4.38	7.31	2.42	1.15	1.20
4	0.050	43.28	2.42	19.45	2.92	99.78	0.13	91.40	1.17	8.35	0.62	1.52	1.80
5	0.100	69.39	1.10	36.52	2.27	99.95	0.03	99.85	0.09	8.16	0.56	2.74	1.36
6	0.500	99.99	0.03	77.92	2.76	99.98	0.01	99.90	0.24	18.76	3.15	10.89	1.56
7	1.000									29.24	0.44	12.67	1.80
8	2.000									97.63	0.10	18.93	0.67
9	3.000									98.31	0.06	95.39	0.14
10	5.000											97.18	0.12

Figure 28. Percent collagenase inhibition as a function of Puag-Haad concentration compared to other antioxidants (mean ± SD, n = 3)



Plot on logarithmic scale

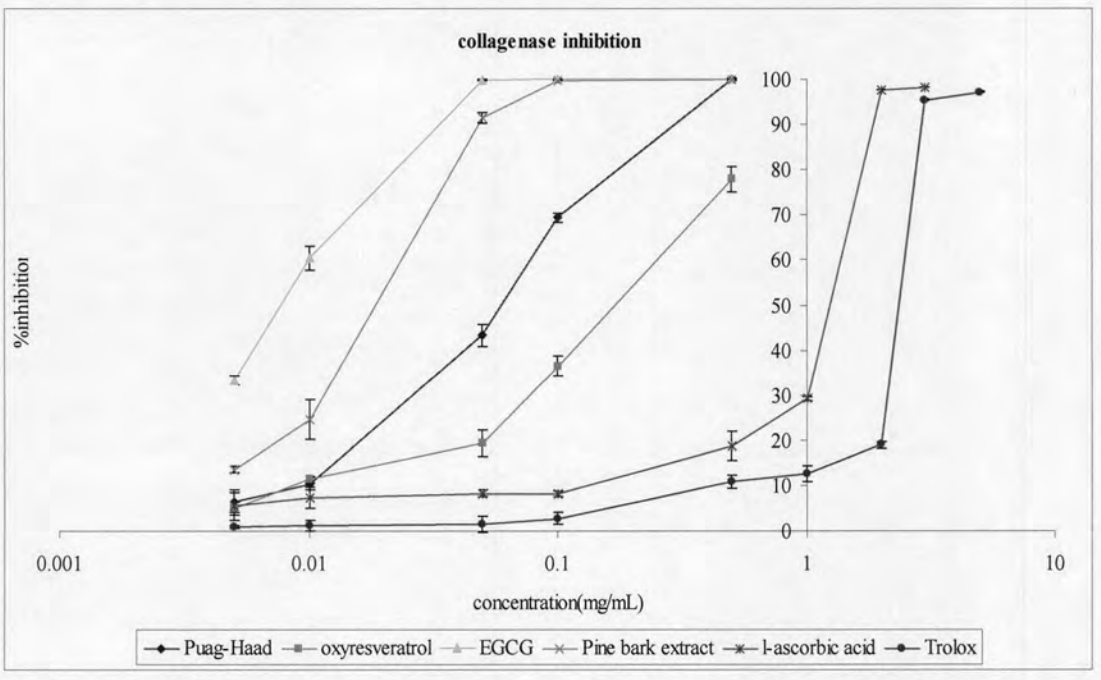


Figure 29 shows the individual plots between % collagenase inhibition and concentration, constructed separately for each antioxidant. The plots clearly indicate that Puag-Haad was more effective than oxyresveratrol in inhibiting collagenase enzyme, with higher extent of inhibition observed up to 0.5 mg/mL concentration.

The inhibition of EGCG and pine bark was apparently more effective than Puag-Haad and oxyresveratrol, reaching nearly 100 % inhibition at very low concentrations of 0.05 and 0.1 mg/mL, respectively.

L-ascorbic acid and Trolox®, on the other hand, was far less effective in inhibiting collagenase, reaching plateau at a much higher concentration of 2 and 3 mg/mL, respectively. The raw data of the absorbance values and % collagenase inhibition of the individual antioxidants are presented in Appendix G.

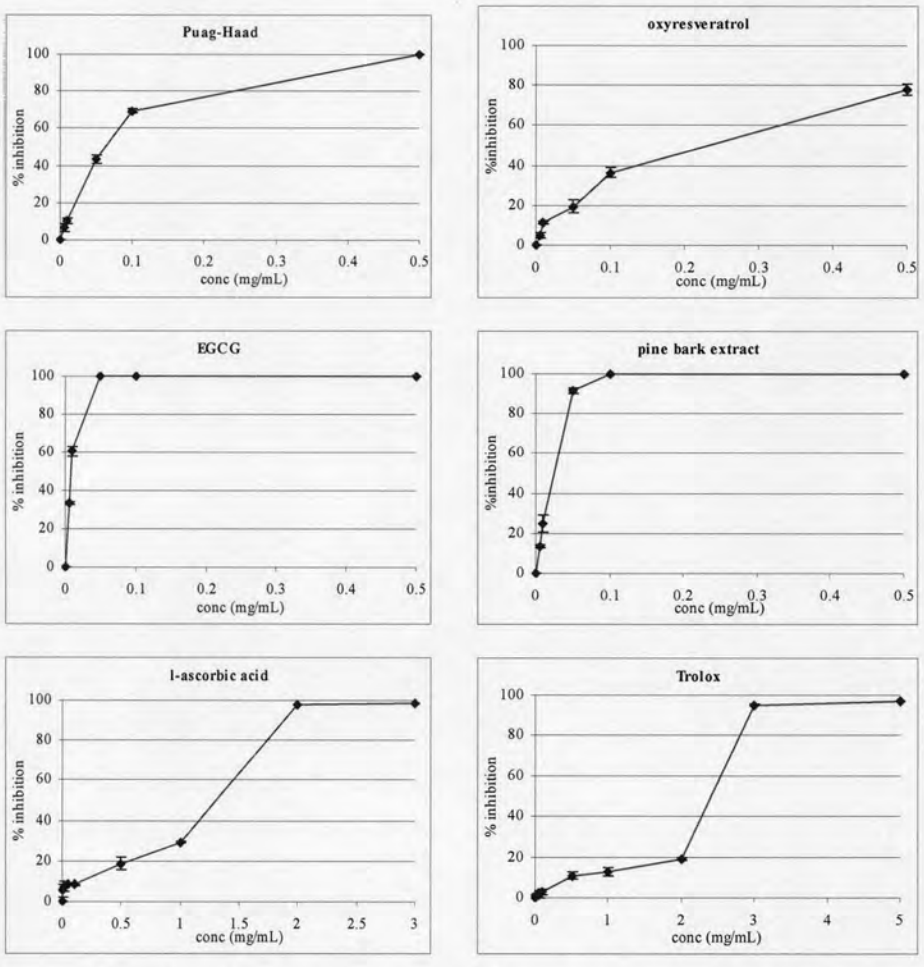


Figure 29. Plots between percent collagenase inhibition and concentration of the individual antioxidants (mean $\pm$ SD, n = 3)

The IC_{50} or a concentration at which an antioxidant can cause 50% inhibition was calculated for each antioxidant by regression analysis of the plot. It was found that a polynomial equation gave a better R^2 (regression coefficient) than a linear model. Moreover, the range of the plot used for calculation was also important. It

was found that the initial range of concentration before reaching plateau gave a highest R^2 of 0.9405 – 0.9999, as shown in Table 19 and Figure 30. Also, the curves obtained from partial polynomial regression were similar to the actual plots. Thus, the polynomial regression equation was used to determine the IC_{50} values for all the tested antioxidants.

Table 19 IC_{50} values of collagenase inhibition of each antioxidant. The regression coefficient (R^2) of the polynomial regression equation between percent inhibition and antioxidant concentration are also provided.

No.	Sample	Polynomial equation (partial)					
		IC_{50} (mg/mL)	Mean	SD	R^2	Mean	SD
1	Puag-Haad	0.0563 0.0641 0.0560	0.0588	0.046	0.9996 0.9960 0.9996	0.9984	0.0021
2	Oxyresveratrol	0.1440 0.1521 0.1632	0.1531	0.0096	0.9933 0.9880 0.9894	0.9902	0.0027
3	EGCG	0.0078 0.0084 0.0077	0.0080	0.0004	0.9999 0.9998 1.0000	0.9999	0.0001
4	pine bark extract	0.0221 0.0249 0.0186	0.0219	0.0032	0.9999 0.9991 0.9999	0.9996	0.0005
5	L-ascorbic acid	1.3629 1.3178 1.3135	1.3314	0.0274	0.9913 0.9867 0.9869	0.9883	0.0026
6	Trolox®	2.3426 2.3420 2.3584	2.3480	0.0090	0.9457 0.9351 0.9406	0.9405	0.0053

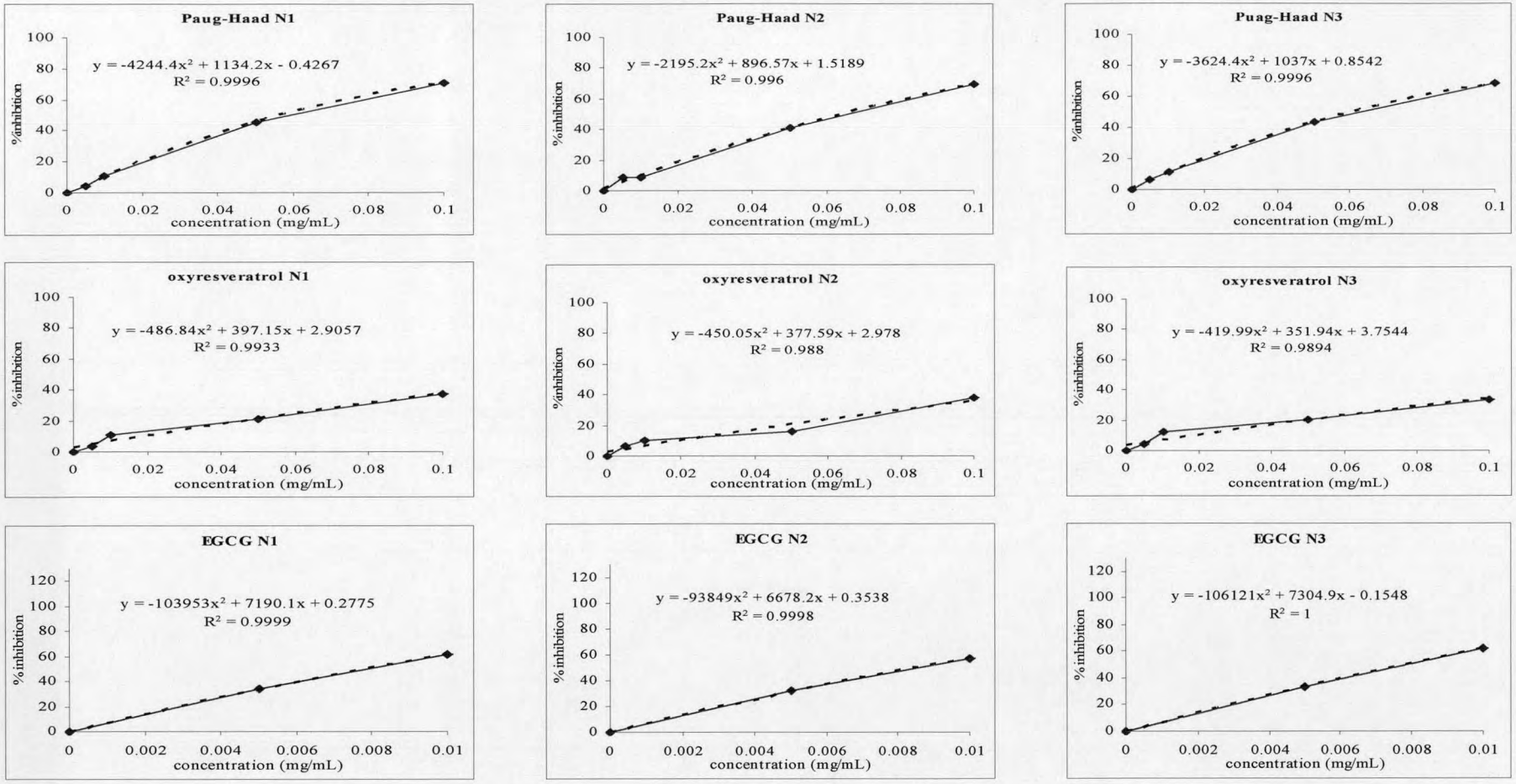


Figure 30. Actual curve (dotted line) and regression curve (solid line) of the initial portion of % collagenase inhibition - concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) values are also provided for the individual antioxidants

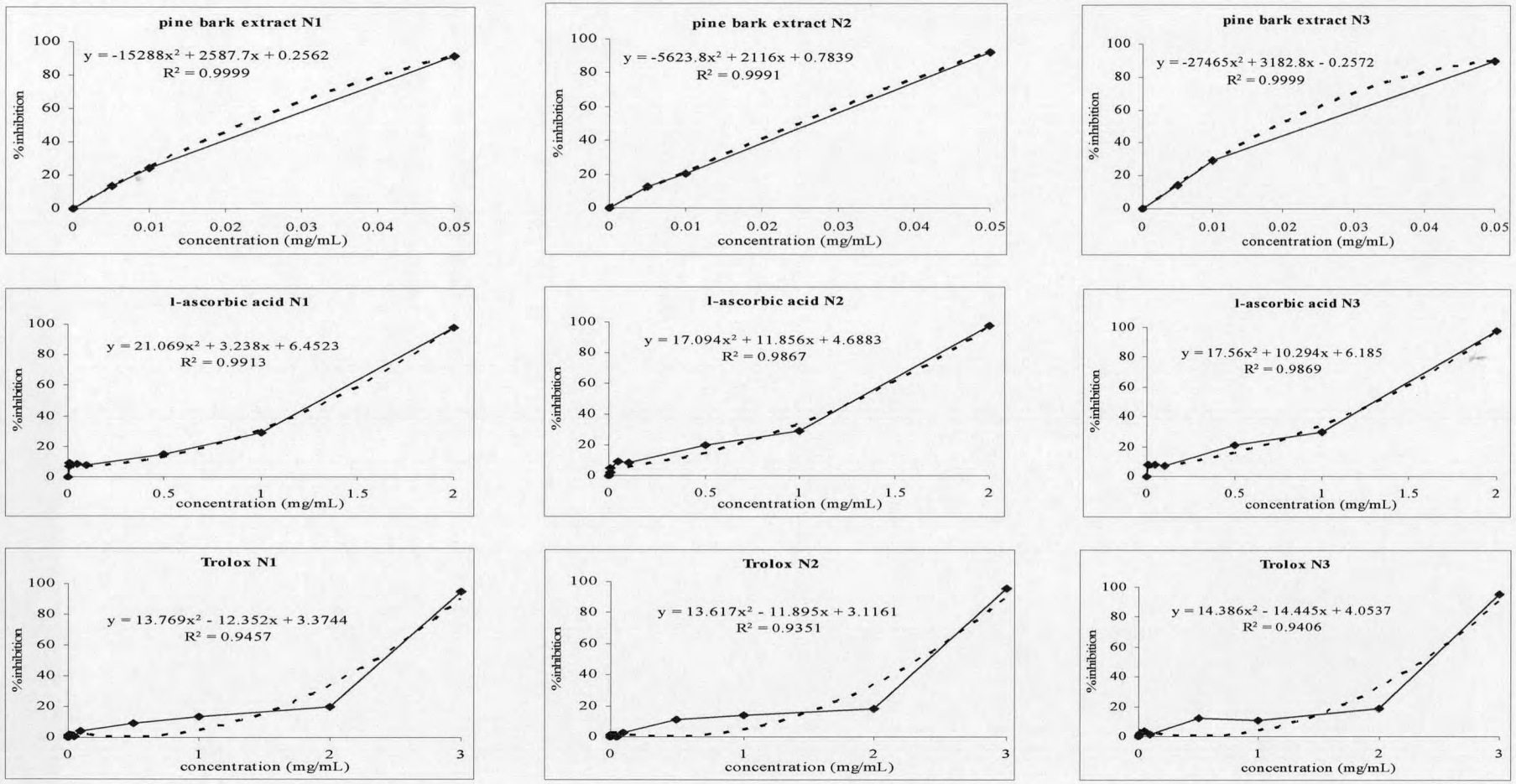


Figure 30 (continued). Actual curve (dotted line) and regression curve (solid line) of the initial portion of % collagenase inhibition - concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) values are also provided for the individual antioxidants

From Table 19, the average values of the estimated IC_{50} from the lowest to the highest value are: EGCG (0.008 $\pm$ 0.0004 mg/mL), pine bark (0.0219 $\pm$ 0.0032 mg/mL), Puag-Haad (0.0588 $\pm$ 0.046 mg/mL), oxyresveratrol (0.1531 $\pm$ 0.0096 mg/mL), L-ascorbic acid (1.3314 $\pm$ 0.0274 mg/mL) and Trolox® (2.348 $\pm$ 0.009 mg/mL). Since IC_{50} value is an indicator of an anti-collagenase potency, Puag-Haad and oxyresveratrol showed weaker collagenase inhibition than EGCG and pine bark because higher concentrations were required for Puag-Haad and oxyresveratrol to give inhibition extent (50% inhibition) similar to the latter two. Due to their very high IC_{50} values, L-ascorbic acid and Trolox® demonstrated a rather weak collagenase inhibitory potency compared to other antioxidants (Figure 31).

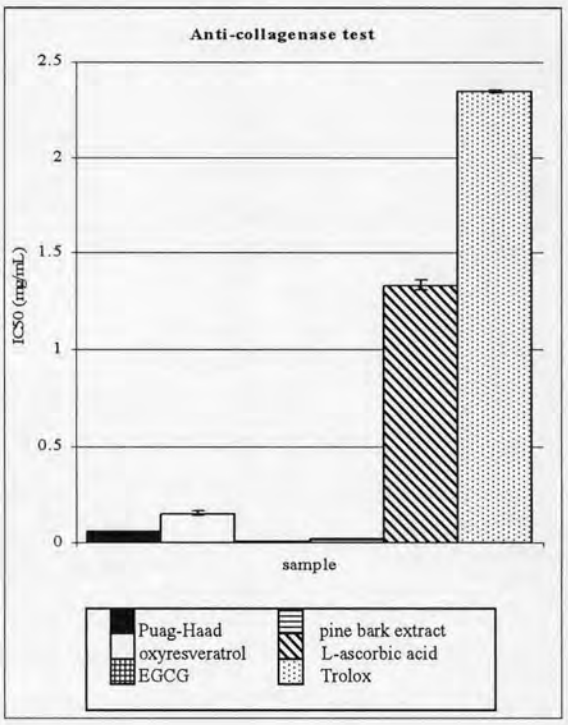


Figure 31. Histogram comparing the mean IC_{50} values (mg/mL) for collagenase inhibition among the six antioxidants (mean $\pm$ SD, n = 3)

The IC_{50} values were analyzed by one-way ANOVA at 5% significant level. They were found to be significantly different ($P << 0.05$). The data were subsequently analyzed by post-ANOVA Tukey's test to rank their anti-collagenase efficacy. The ranking results showed that the antioxidants could be divided into 5 groups with different collagenase inhibitory potency ($P < 0.05$). The ranking of IC_{50}, from the

most potent collagenase inhibitor (smallest IC_{50} value) to the least potent inhibitor (largest IC_{50} value) was as follows:

EGCG ≈ pine bark	<	Puag-Haad	<	oxyresveratrol	<	L-ascorbic acid < Trolox®
0.01		0.02		0.06		0.15 1.33 2.35 mg/mL

The antioxidants that are joined by the same underline are within the same group and not statistically different in term of their collagenase inhibition ($P > 0.05$). Thus, both the EGCG and pine bark extract demonstrated the highest potency of anti-collagenase activity with the smallest IC_{50} values. Their potency was significantly greater than other antioxidants ($P > 0.05$). Puag-Haad was the second most potent collagenase inhibitor, with mean IC_{50} of 0.06 mg/mL, which was more potent than oxyresveratrol (IC_{50} 0.15 mg/mL), which in turn was more potent than L-ascorbic acid (IC_{50} 1.33 mg/mL). Trolox©, with the highest IC_{50} of 2.35 mg/mL, was the least effective inhibitor ($P < 0.05$). The statistical results of the individual antioxidants are shown in Appendix G.

Figure 32 also shows histograms comparing % collagenase inhibition of the six antioxidants at each concentration. Similar to the IC_{50} values, EGCG showed the highest inhibition percentage especially at very low concentrations, whereas Puag-Haad and oxyresveratrol had intermediate effect. L-ascorbic acid and Trolox© always gave much lower inhibition extent at all concentrations in agreement with their very high IC_{50} values. It should be noted, however, that solutions of L-ascorbic acid and Trolox© were more difficult to dissolve, especially at 2 mg/mL and above. This may have contributed to the variation in the measurement of % collagenase inhibition and IC_{50} values since a clearly soluble solution could not be obtained at higher concentrations. Nevertheless, the two were still considered the least effective inhibitors since at concentrations below 2 mg/mL, where clear solutions were obtained, their extent of enzyme inhibition was already the lowest. Thus, the data from this study revealed that both Puag-Haad and oxyresveratrol were able to inhibit collagenase type IV, a major enzyme responsible for the degradation of collagen and other structural proteins which leads to skin aging. Further studies are needed to determine their possible inhibitory effects on other types of collagenases.

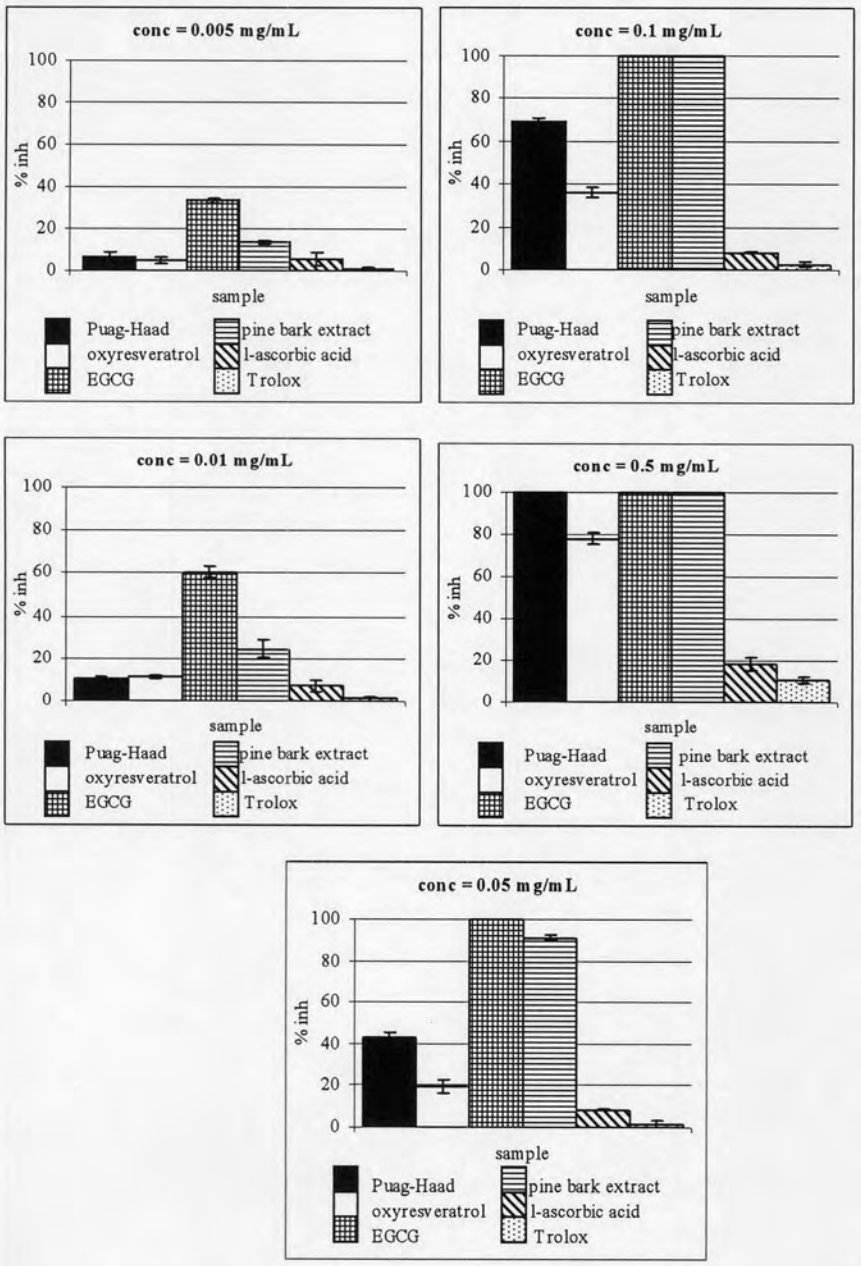


Figure 32. Histograms comparing percent collagenase inhibition of the six antioxidants at each concentration (mean $\pm$ SD, n = 3)