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Case Study

In Vitro Assessment of the Anti-Oxidant Potential of Five Active Cosmetic Ingredients in the Development of an Anti-Ageing Skincare Formulation

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Abstract

Anti-oxidant activity forms a vital part of the body's natural defence system against reactive oxygen species (ROS), which are formed as a by-product of metabolism and can be harmful to cells. ROS levels in the skin are increased dramatically by a variety of internal and external factors including stress, the ageing process and exposure to air pollutants. Anti-oxidant cosmetic ingredients may therefore support a variety of claims including anti-pollution and anti-ageing and have gained significant popularity. The OxiSelect™ test measures anti-oxidant activity against hydroxyl, peroxyl and other ROS in human skin cell (epidermal keratinocyte) cultures, indicating the potential of active cosmetic ingredients to protect against the effects of ageing and pollution. The aim of this study was to assess the anti-oxidant potential of five active ingredients (designated codes A, B, C, D, E), alone and in combination (A+B; A+C; A+B+C; A+B+D; A+B+E in equal concentrations). Two different concentrations (0.01% and 0.05%) of each test item and combinations were tested and compared to the untreated negative control (NC) and positive control (quercetin). The results placed the 10 conditions into a rank order of activity, and demonstrated that all test items at the highest concentration (0.05%) have anti-oxidant properties. Ingredient B was the most potent of the five, and its activity was further increased when used in combination with ingredients A and E together, demonstrating a synergistic effect. The results were used to create a cosmetic formulation (anti-ageing skincare product) with optimal anti-oxidant activity.

Introduction

Oxygen is an essential requirement for life, but it is also a very reactive molecule and can be harmful when converted to reactive oxygen species (ROS) within the body. ROS are unstable and react with most biological molecules, causing damage to vital cellular components such as DNA, proteins and lipids, contributing to inflammation and ageing. Stress and exposure to air pollutants can dramatically increase ROS levels in the skin and lead to an acceleration of the inflammatory and ageing pathways[1]. Our cells are equipped with natural anti-oxidant enzymes to prevent ROS from forming, or to remove them before they cause damage. Providing our skin with an anti-oxidant boost through active ingredients in cosmetic products can help to enhance the skin's own natural protective mechanisms[2]. Anti-oxidant ingredients may therefore support a variety of claims including anti-pollution and anti-ageing.

The OxiSelect™ test[3] measures anti-oxidant activity against hydroxyl, peroxyl and other reactive oxygen species (ROS) in human epidermal keratinocyte cultures, indicating the potential of active cosmetic ingredients to protect against the effects of ageing and pollution. At XCellR8, human keratinocytes are grown in animal-product-free conditions, maximising the relevance of the test system to human physiology and providing additional ethical advantages by avoiding the use of any animal-derived components (Figure 1)[4,5]. The OxiSelect™ test employs the cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). The probe diffuses into cells and is deacetylated by cellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescein (DCFH), which is rapidly oxidised to the highly fluorescent 2',7'-Dichlorohydrofluorescein (DCF) by reactive oxygen species (ROS) in the presence of the proprietary "Free Radical Initiator" (Figure 2). The fluorescence intensity is proportional to the levels of ROS within the cell cytosol, and is therefore reduced in the presence of anti-oxidant active ingredients. The positive control, quercetin, is a plant flavonoid widely found in fruits and vegetables. It inhibits the formation of free radicals and thus DCF formation in a dose-dependent manner.

The aim of this study was to assess the anti-oxidant potential of five novel active ingredients (designated codes A, B, C, D, E), alone and in combination (A+B; A+C; A+B+C; A+B+D; A+B+E in equal concentrations), as part of the product development process for an innovative anti-ageing skincare formulation.

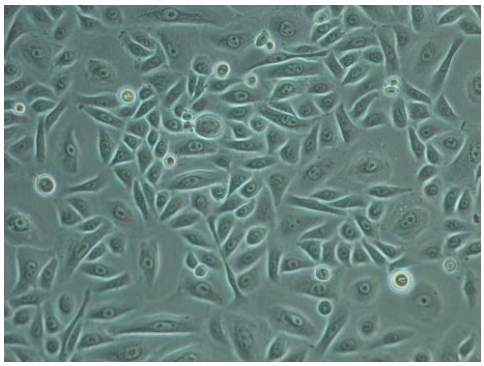


Figure 1: Keratinocytes derived from the epidermis of human skin and grown in animal-product-free culture conditions. Cells were obtained commercially with full donor consent, comprehensive health screening and QC testing prior to use.

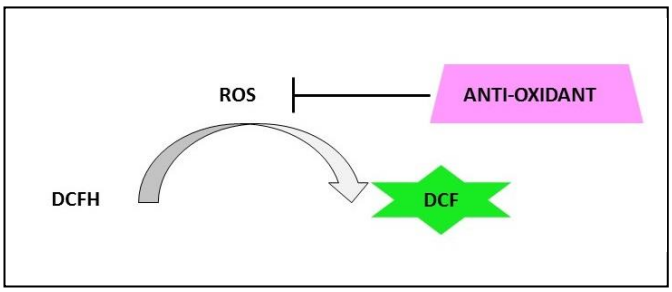


Figure 2: The OxiSelect™ test uses the fluorogenic probe 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). The probe diffuses into cells and is converted by esterases to 2', 7'-Dichlorodihydrofluorescein (DCFH), which is rapidly oxidised to the highly fluorescent metabolite 2',7'-Dichlorohydrofluorescein (DCF) by reactive oxygen species (ROS) in the presence of the proprietary "Free Radical Initiator". Fluorescence intensity is reduced (compared with controls) in the presence of anti-oxidant cosmetic ingredients.

Method

Solubility: The solubility of E, G, R, P and D in cell culture medium was determined as per XCellR8's standard operating procedure, which follows the guidance set out in OECD Guidance Document 129[6]. When test items are not soluble in cell culture medium, the SOP details a procedure for assessing solubility in solvents (starting with DMSO, then ethanol), followed by dilution into cell culture medium.

Cytotoxicity: Human epidermal keratinocyte cultures were grown until 80-90% confluent in 96-well culture plates, in animal product-free conditions, and then exposed to each active ingredient alone, and in the 5 combinations listed in Table 1, for 2 hours. The concentrations tested for each condition covered the requested range to be used in the anti-oxidant tests and were compatible with the solubility assessment. Cell viability relative to untreated negative controls was assessed using the MTT test (an indicator of metabolic capacity). The positive control was the non-ionic surfactant Triton X-100 and the negative control was culture medium. The cell viability results for the active ingredients indicated the concentration range that could be tolerated by the cell cultures during the main anti-oxidant study.

Anti-Oxidant Test: Anti-oxidant activity was assessed using the OxiSelect™ method[3]. Human epidermal keratinocytes were cultured until 80-90% confluent in 96-well culture plates, in animal product-free culture conditions. Culture medium was then removed and the cells washed with phosphate buffered saline (PBS). DCFH-DA probe solution was added to the cells along with quercetin (positive control), or the active ingredients, dissolved in cell culture medium, according to the agreed non-cytotoxic concentrations as shown in Table 1. Each condition was tested in triplicate. After incubation for 60 minutes at 37°C, cells were rinsed and Free Radical Initiator solution was added to the wells. Fluorescence intensity was read immediately and monitored over a 60-minute period, in increments of 5 minutes. The excitation and emission wavelengths were 480nm and 530nm respectively. Results were plotted in the graph format and statistical significance of the results was determined using the Student's t-test.

Test Item	Test concentration	Administration method
A	0.05% and 0.01%	Cell culture medium
B	0.05% and 0.01%	Cell culture medium
C	0.05% and 0.01%	Cell culture medium
D	0.05% and 0.01%	Cell culture medium
E	0.05% and 0.01%	Cell culture medium
A+B	0.05%E + 0.05%G; 0.01%E + 0.01%G	Cell culture medium
A+C	0.05%E + 0.05%R; 0.01%E + 0.01%R	Cell culture medium
A+B+C	0.05%E +0.05%G +0.05%R; 0.01%E + 0.01%G +0.01%R	Cell culture medium
A+B+D	0.05%E + 0.05%G + 0.05%P; 0.01%E + 0.01%G +0.01%P	Cell culture medium
A+B+E	0.05%E + 0.05%G +0.05%D; 0.01%E + 0.01%G +0.01%D	Cell culture medium

Table 1: Concentrations of the five cosmetic active ingredients, alone and in combination, in the OxiSelect™ test. Each condition was tested in triplicate.

Results

In all experiments, positive and negative controls performed within the defined acceptance criteria, confirming the validity of the test. Figure 3 demonstrates the dose-dependent anti-oxidant effect of the positive control, quercetin. Note the inverse relationship between anti-oxidant activity and fluorescence, since anti-oxidant ingredients inhibit conversion of the substrate (DCFH) to its fluorescent metabolite (DCF). Therefore, the lowest fluorescent signal is seen using the highest concentration of quercetin. The same principle applies to Figure 4, which demonstrates that a dose-dependent anti-oxidant activity was observed for all five cosmetic active ingredients, both alone and in combination. Statistical significance of the results was determined using the Student's t-test. The levels of significance ($p<0.05$, $p<0.01$ and $p<0.001$) are marked in Table 2, which also shows the rank order of anti-oxidant activity in the 10 different combinations of active ingredients.

The results demonstrate the presence of anti-oxidant activity in all ingredients, but to varying extents, with some synergistic effects observed when the ingredients were combined. The observations shown in Figure 4 can be summarised as follows: **Ingredient A** shows a statistically significant anti-oxidant effect at the highest tested concentration (0.05%). This effect is similar to quercetin at 31.3µM. **Ingredient B** shows a statistically significant anti-oxidant effect at both concentrations. The effect with 0.01% is similar to quercetin at 31.3µM, while the effect with 0.05% is **even better** than quercetin at 31.3µM. Ingredient B was the most potent of the five when used alone. **Ingredient C** shows a statistically significant anti-oxidant effect at the highest tested concentration (0.05%). This effect is similar to quercetin at 31.3µM. **Ingredient D** shows a statistically significant anti-oxidant effect at both concentrations. The effect at 0.05% is **even better** than quercetin at 31.3µM. **Ingredient E** shows a statistically significant anti-oxidant effect at the highest tested concentration (0.05%). This effect is similar to quercetin at 31.3µM. **The combination of ingredients A+B** shows a statistically significant anti-oxidant effect at both concentrations. At 0.05%, this effect is **even better** than quercetin at 31.3µM. The combination showed a higher level of activity than ingredient A alone, and was similar to ingredient B alone. **The combination of ingredients A+C** shows a statistically significant anti-oxidant effect at the highest tested concentration (0.05%). This effect is lower than quercetin at 31.3µM. This combination of ingredients was no different in potency to ingredients A or C used alone, so no synergistic effect was observed. **The combination of ingredients A+B+C** shows a statistically significant anti-oxidant effect at both concentrations. At 0.05% this effect is **even better** than quercetin at 31.3µM. This combination of ingredients was more potent than A and C alone, but similar to B alone. **The combination of ingredients A+B+D** shows a statistically significant anti-oxidant effect at both concentrations. At 0.05% this effect is **even better** than quercetin at 31.3µM. This combination of ingredients was more potent than A alone and similar to D alone. **The combination of ingredients A+B+E** shows a statistically significant anti-oxidant effect at both concentrations. The 2 concentrations are **even better** than quercetin at 31.3µM. This combination showed the highest anti-oxidant activity observed in the study, and was more potent than ingredients A or E alone, but similar to B alone.

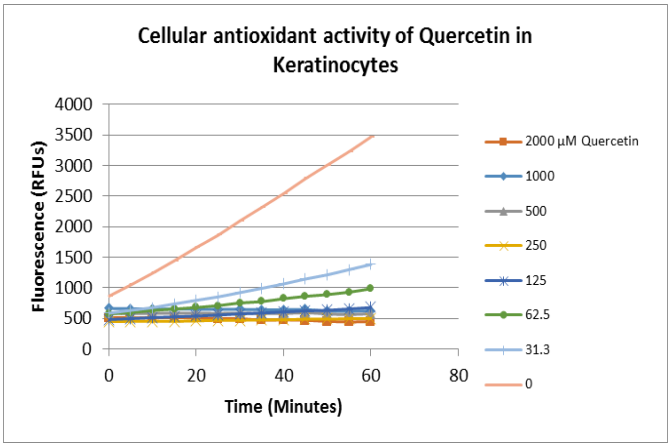
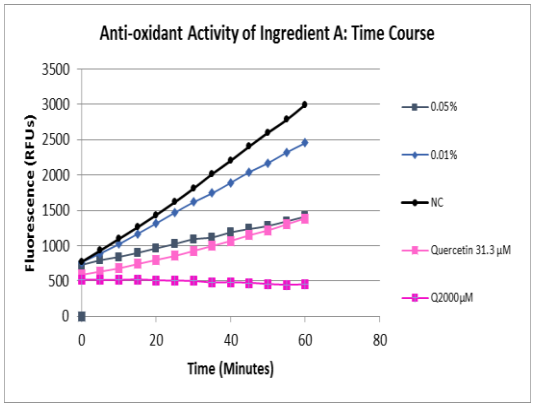
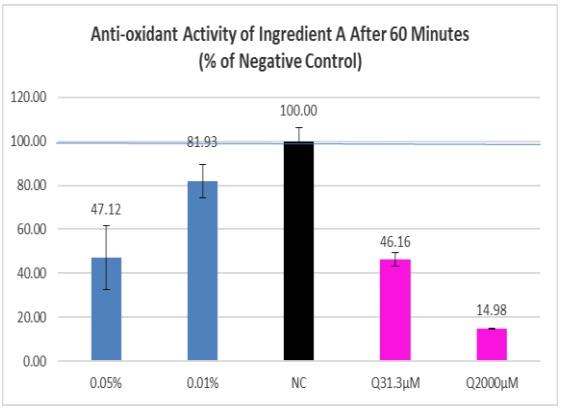
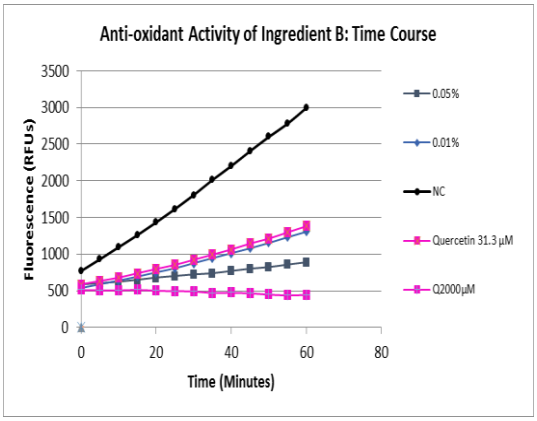


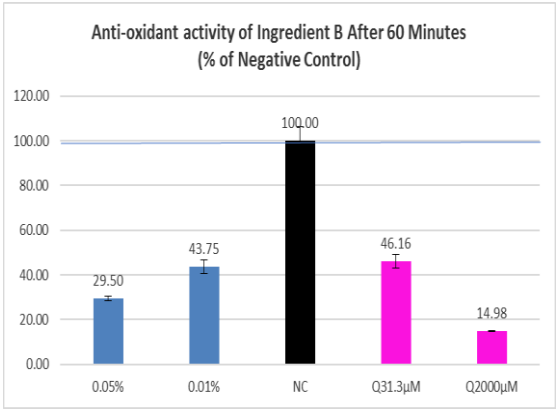
Figure 3: Anti-oxidant activity of quercetin (positive control) in human epidermal keratinocyte cultures following 1 hour incubation

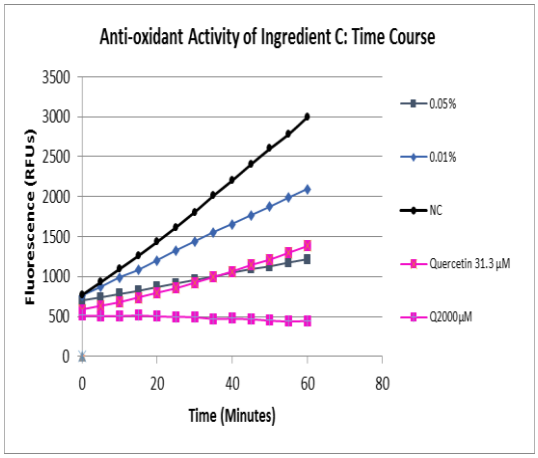
Figure 4: Anti-oxidant activity of the five active ingredients, alone and in combination. Fluorescence was measured every 5 minutes for 60 minutes. In black, the negative control (NC, untreated cells) where the fluorescence increases over time. In pink, the positive control (quercetin) at 2 concentrations. In blue, the active ingredient(s), at 2 concentrations. Error bars denote the standard deviation of the mean for n=3 samples. Statistical significance of the results was determined using the Student's t-test and the significance levels are included in Table 2.

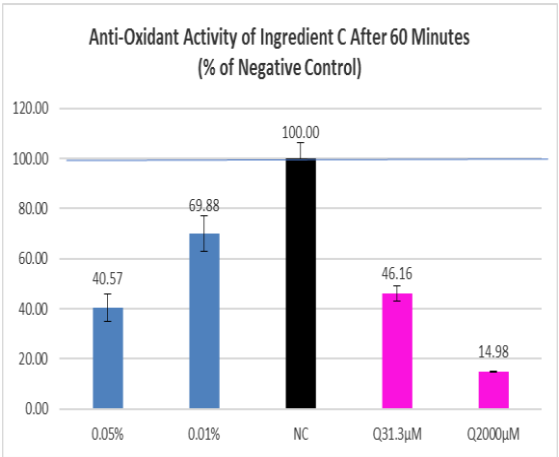


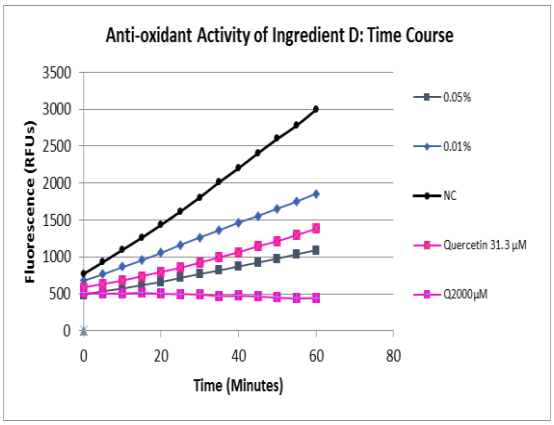


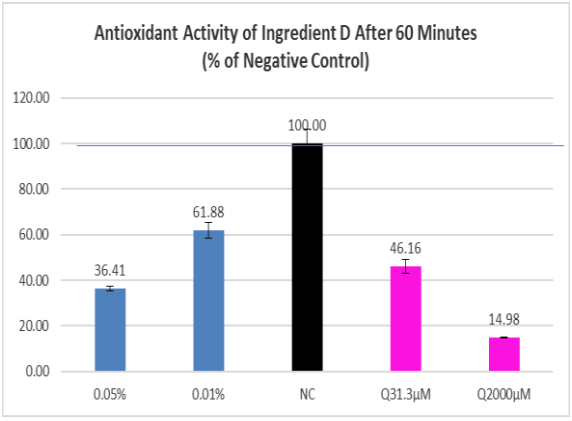


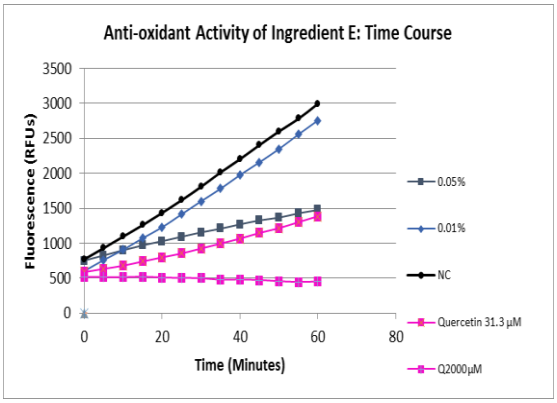


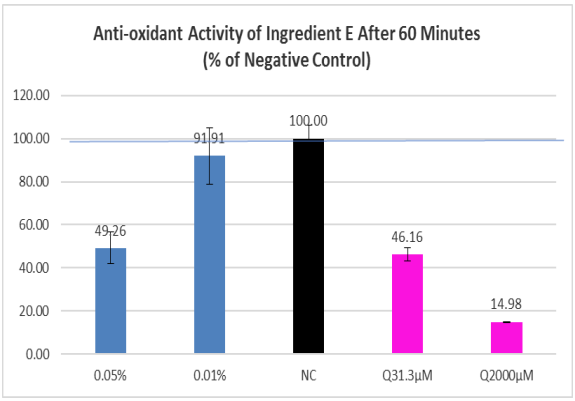


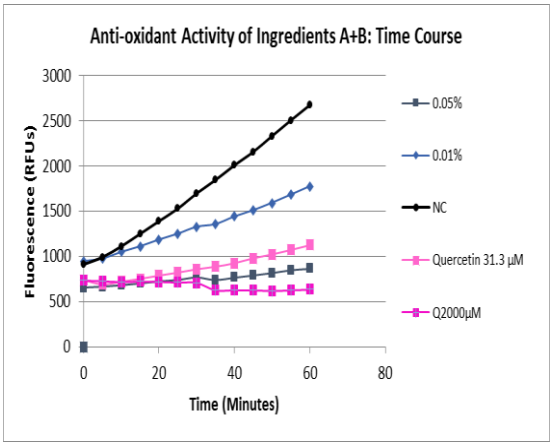


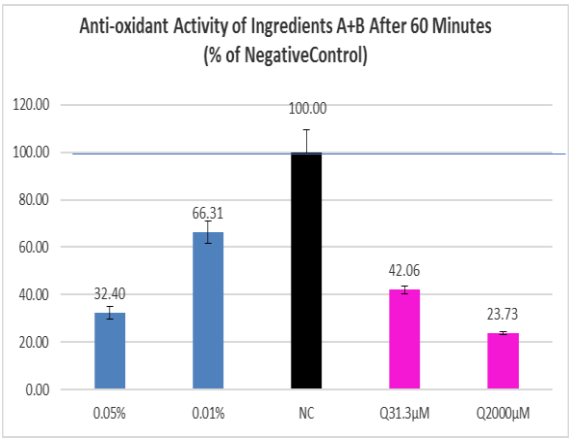

Anti-oxidant Activity of Ingredient D: Time Course
Fluorescence (RFUs)
Time (Minutes)
0.05%
0.01%
NC
Quercetin 31.3 µM
Q2000µM

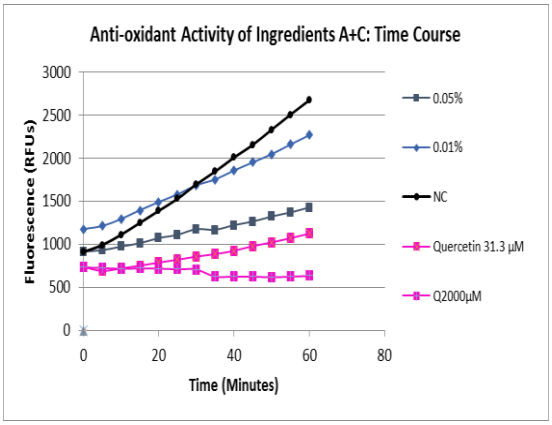

Antioxidant Activity of Ingredient D After 60 Minutes
(% of Negative Control)
36.41
61.88
100.00
46.16
14.98
0.05%
0.01%
NC
Q31.3µM
Q2000µM

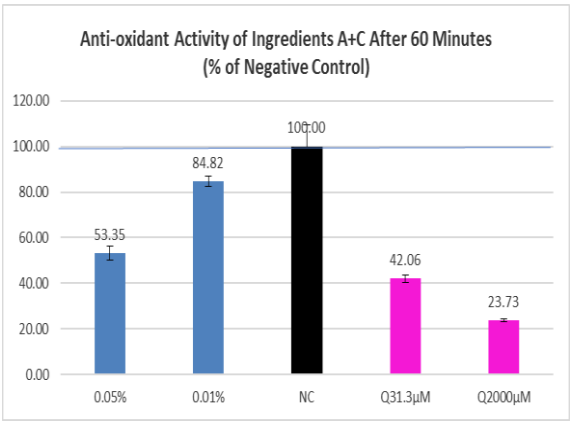

Anti-oxidant Activity of Ingredient E: Time Course
Fluorescence (RFUs)
Time (Minutes)
0.05%
0.01%
NC
Quercetin 31.3 µM
Q2000µM

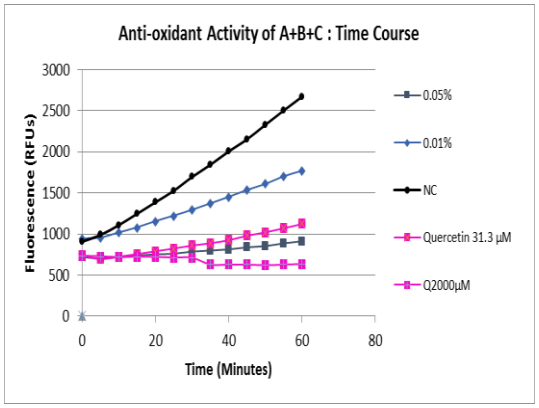

Anti-oxidant Activity of Ingredient E After 60 Minutes
(% of Negative Control)
49.26
91.91
100.00
46.16
14.98
0.05%
0.01%
NC
Q31.3µM
Q2000µM

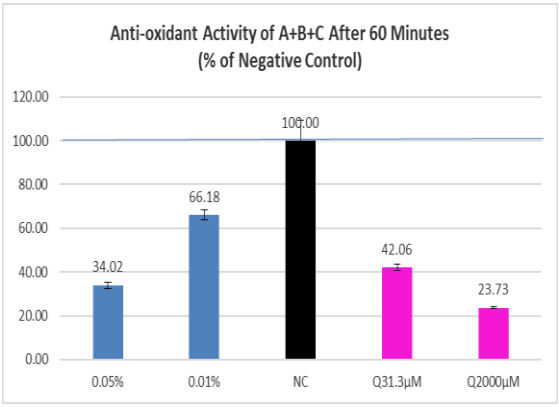

Anti-oxidant Activity of Ingredients A+B: Time Course
Fluorescence (RFUs)
Time (Minutes)
0.05%
0.01%
NC
Quercetin 31.3 µM
Q2000µM

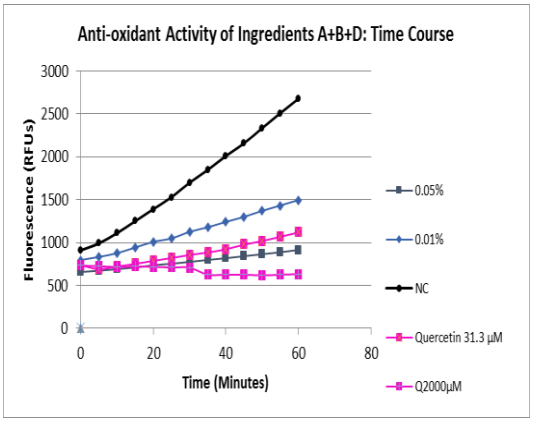

Anti-oxidant Activity of Ingredients A+B After 60 Minutes
(% of NegativeControl)
32.40
66.31
100.00
42.06
23.73
0.05%
0.01%
NC
Q31.3µM
Q2000µM

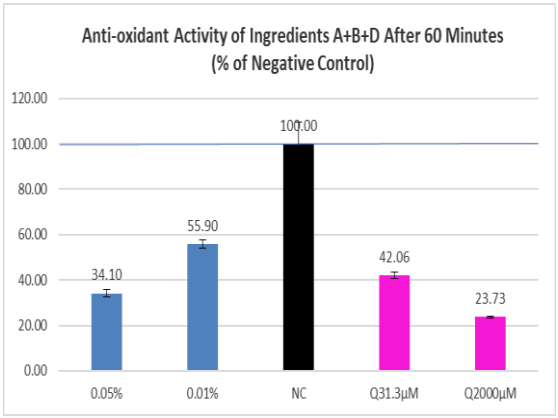

Anti-oxidant Activity of Ingredients A+C: Time Course
Fluorescence (RFUs)
Time (Minutes)
0.05%
0.01%
NC
Quercetin 31.3 µM
Q2000µM

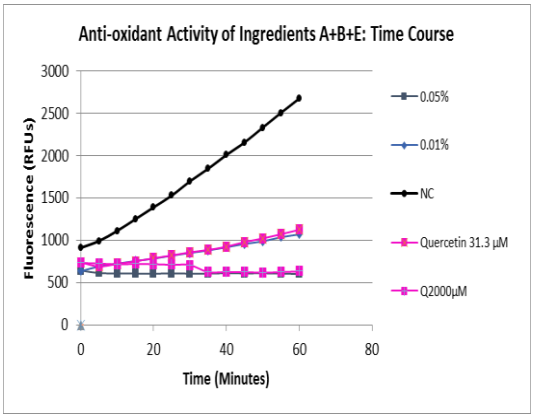

Anti-oxidant Activity of Ingredients A+C After 60 Minutes
(% of Negative Control)
53.35
84.82
100.00
42.06
23.73
0.05%
0.01%
NC
Q31.3µM
Q2000µM

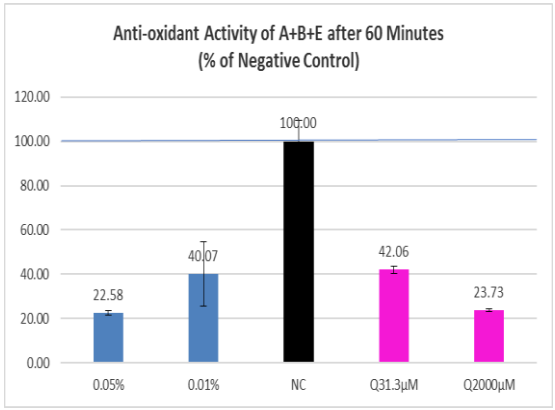












Test Item	Anti-oxidant activity (% of negative control)		Rank order of activity (1 = highest)
	Test concentration (% in cell culture medium)		
	0.01%	**0.05%**	
A	81.93%	47.12%**	8
B	43.75%**	29.50%***	2
C	69.88%	40.57%**	7
D	61.88%*	36.41%***	6
E	91.91%	49.26%**	9
A+B	66.31%*	32.40%***	3
A+C	84.82%	53.35%**	10
A+B+C	66.18%*	34.02%***	4
A+B+D	55.90%**	34.10%***	5
A+B+E	40.07%***	22.58%***	1

Table 2: Summary of results and rank order of anti-oxidant activity for the 10 different combinations of active ingredients (mean of n=3 samples). Asterisks denote statistical significance using the "Student's t-test": * p<0.05, ** p<0.01, *** p<0.001.

Discussion

In this study, the anti-oxidant properties of five cosmetic active ingredients, alone and in combination, were evaluated at 2 concentrations (0.01% and 0.05%) in human keratinocytes and compared to the plant flavonoid, quercetin, as a positive control. A clear dose-response over a range of concentrations of quercetin (Figure 3) demonstrated the validity of the study. The results (Figure 4 and Table 2) also confirm the presence of statistically significant anti-oxidant activity in all five ingredients, with a rank order of activity as follows: B > D >C > A > E (based on activity at a test concentration of 0.05% when the five ingredients were tested alone). The minimum level of statistical significance observed at this concentration was $p<0.01$. All ingredients demonstrated a dose-response anti-oxidant effect, with higher activity when used at a concentration of 0.05% compared with 0.01%. Ingredient B was the most potent of the five, and its activity was further increased when used in combination with ingredients A and E together, demonstrating a synergistic effect. A+B+E was overall the most potent combination tested.

The results of this study were applied in the development of an anti-ageing skincare formulation with optimal anti-oxidant activity.

References

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