Erythrocytes and cell line-based assays to evaluate the cytoprotective activity of antioxidant components obtained from natural sources

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Abstract

Oxidative stress can damage cellular components including DNA, proteins or lipids, and may cause several skin diseases. To protect from this damage and addressing consumer's appeal to natural products, antioxidants obtained from algal and vegetal extracts are being proposed as antioxidants to be incorporated into formulations. Thus, the development of reliable, quick and economic *in vitro* methods to study the cytoactivity of these products is a meaningful requirement.

A combination of erythrocyte and cell line-based assays was performed on two extracts from *Sargassum muticum,* one from *Ulva lactuca,* and one from *Castanea sativa*. Antioxidant properties were assessed in erythrocytes by the TBARS and AAPH assays, and cytotoxicity and antioxidant cytoprotection were assessed in HaCaT and 3T3 cells by the MTT assay. The extracts showed no antioxidant activity on the TBARS assay, whereas their antioxidant capacity in the AAPH assay was demonstrated. On the cytotoxicity assays, extracts showed low toxicity, with IC_{50} values higher than 200 µg/mL. *C. sativa* extract showed the most favourable antioxidant properties on the antioxidant cytoprotection assays; while *S. muticum* and *U. lactuca* extracts showed a low antioxidant activity. This battery of methods was useful to characterize the biological antioxidant properties of these natural extracts.

Keywords

Antioxidant extract; haemolysis; cytotoxicity; TBARS; AAPH

Abbreviations

AAPH: 2,2-azobis(2-amidinopropane) dihydrochloride

CmaxA-OX: Concentration with a maximal antioxidant effect.

DMEM: Dubecco's Modified Eagle's Medium

DPPP: Diphenyl-1-pyrenylphosphine

FBS: Foetal Bovine Serum

MAC: Maximal Antioxidant Concentration

MTT: 3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide

ROS: Reactive oxygen species

TBARS: Thiobarbituric acid reactive species

Introduction

Living cells and organisms are exposed to oxidative stress by reactive oxygen species (ROS), produced either by physiological or exogenous processes. Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism. At low to moderate concentrations, they function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components, such as lipids, proteins, and DNA. The shift in balance between oxidant/antioxidant in favor of oxidants is termed "oxidative stress." Oxidative stress contributes to many pathological conditions, including cancer, neurological disorders, Aerobic organisms have integrated antioxidant systems, which include enzymatic and nonenzymatic antioxidants that are usually effective in blocking harmful effects of ROS.

Exogenous factors include UV radiation, a number of toxins, and many xenobiotics from diet or other exposure events, either voluntary or not; endogenous sources of oxidative stress are of similar variability. Almost every cell structure could be targeted by ROS: membrane phospholipids, proteins and nucleic acids can suffer modifications which can, to a variable extent, compromise cell viability or disrupt important processes. Problems in genetic material, loss of function of enzymes or structural proteins and peroxidation of membrane lipids are some of, but not all, the dysfunctions that may arise. Depending on the intensity of the exposure and its derived effects, an arrest in cell cycle, senescence, apoptosis or necrosis could be observed (Chen et al., 2012).

Reactive oxygen species (ROS) have been regarded as harmful molecules that damage various molecules inside cells by oxidation and are responsible for ageing and various human diseases. However, recent studies have revealed an opposite aspect of ROS that these are actively generated in cells and mediate physiological intracellular signalling as second messengers. (Miki and Funato 2012)

Skin is a large organ which, according to its anatomic location, may suffer from important oxidative stress. It directly receives UV radiation, and it can also be exposed to many environmental toxics. The long term consequences may include photoageing, immunosupression or even malignant processes (Halliday et al., 2012).

Antioxidants prevent or revert to the former situation the effects induced by oxidative stress. Due to the great number and diversity of these effects, a great industrial interest exists in researching new antioxidants, especially in dermopharmaceutic and cosmetic industries. Along with consumer trends' demands of natural products, many plant and algae-derived products are being investigated as ingredients for potential use in cosmetics, dermopharmacy, food and other aspects of healthcare, either as active ingredients or as excipients (Shalaby, 2011).

Due to the animal ban in the assessment of new ingredients and cosmetic products in Europe, the development of reliable, quick and economic *in vitro* methods is a meaningful requirement for researchers. Such methods can be classified into three groups: chemical methods, erythrocyte-based methods and cell line-based methods (Chen et al., 2011; Ugartondo et al., 2009).

The aim of this work was to develop a good strategy to study the antioxidant potential of vegetal extracts obtained from different sources

Therefore, for the judicious choice of antioxidant compounds, here we proposed a battery of assays to analyze the effect on their antioxidant behavior. For this purpose, we (1) induced oxidative stress by H_2O_2 in intact human erythrocytes and in nontumoral cell cultures; (2) analyzed the markers of oxidative stress, namely, hemolysis, lipid peroxidation, and cytotoxicity; and (3) tested the protective capacity of the extracts against oxidative stress.

Materials and methods

Chemicals

Hydrogen peroxide 30% w/w solution sodium azide, malondialdehyde, MTT and 2-thiobarbituric acid were purchased from Sigma (St. Louis, USA). Trichloroacetic acid 20% w/v solution was purchased from Scharlau (Sentmenat, Spain).

Extracts

Four extracts were evaluated: an acidic aquous extract of *Ulva lactuca* (ULE), a cosmopolitan chlorophyte used as food by certain Asian cultures (Tabarsa et al., 2012); a *Castanea sativa* (Chestnut) bur purified extract (CBPE) obtained by non-isothermal autohydrolysis followed by extraction with ethyl acetate and hydroalcoholic washing (Conde et al., 2011a); and two extracts of

Sargassum muticum (SME1 and SME2), a phaeophycian algae of Asian origin. SME1 was obtained by autohydrolysis at 220 ºC followed by ethanol extraction, whereas SME2 was obtained by autohydrolisis at 190 ºC and further extracted with ethanol and formaldehyde.

Erythrocyte-based assays to study the antioxidant properties of the extracts

Erythrocyte suspensions were obtained from Wistar rats anti-coagulated blood as previously described by our group (Martinez et al., 2012); The antioxidant activity of the extracts was studied by the AAPH assay. AAPH (2,2-azobis(2-amidinopropane) dihydrochloride) spontaneously releases ROS, which cause haemolysis of the red blod cells unless antioxidant protection is provided by products tested (Miki et al. 1987). A 12.5% erythrocyte suspension (250 µL) was incubated for 90 min at 37 °C in a shaker in the presence of AAPH at a final concentration of 150 mM, to achieve maximal hemolysis. The same test was performed to detect the antihemolytic activity of the extracts. Concentrations ranging between 15 and 10,000 µg/mL of the compounds dissolved in PBS were added to the erythrocyte suspension in the presence of 150 mM AAPH at 37 °C for 90 min. Erythrocyte controls were included in all of the assays to detect spontaneous haemolysis in the absence of oxidant agent or products. After the incubation time, cells were centrifuged and haemolysis was determined spectrophotometrically at 540 nm (release of haemoglobin). The percentage of haemolysis was calculated by comparing the absorbance (540 nm) of the supernatant of the samples with that of a control sample totally hemolyzed with distilled water. The IC50 (50% inhibitory concentration) of the hemolysis induced by H_2O_2 was determined for the compounds (Ugartondo et al., 2009).

Lipid peroxidation mediated by H_2O_2 led to malondialdehyde (MDA) production, which was indirectly measured by spectrophotometric method determining the thiobarbituric acid reactive (TBAR) substances. The principle of this method depends on extraction of MDA from erythrocyte suspension by trichloroacetic acid (TCA) solution and the subsequent reaction of this MDA with thiobarbituric acid (TBA), which yields a pink coloured complex (maximum absorption at 532 nm) (Stocks and Dormandy, 1972). To induce lipid peroxidation, RBCs were incubated under the same conditions as the hemolysis assay (i.e. with H_2O_2 20 mM alone or with different concentrations of test compound at 37ºC for 90 min). Following incubation, the RBC suspension was mixed with 1 mL of

trichloroacetic acid solution 20% w/v (TCA) to remove potentially interfering substances (Srour et al., 2000). Samples were then centrifuged and 1 mL of supernatant was mixed with 1 mL of 1% 2-thiobarbituric acid (TBA). Finally, samples were heated at 90 ºC for 50 min, cooled and centrifuged before measuring the absorbance of the supernatant 532 nm and 600 nm to discard possible interferences. The appropriate blanks and controls were run alongside the test samples. The degree of lipid peroxidation was expressed in arbitrary absorbance units after subtracting the absorbance of controls.

Previously to the incubation, sodium azide at 2 mM in PBS was added to the cell suspension and was preincubated for 15 min in continuous rotation to enable inactivation of erythrocyte catalase.

Cytotoxicity of extracts in HaCaT keratinocytes and 3T3 fibroblasts

The mouse embryonic cell line 3T3 and the spontaneously immortalized human keratinocyte cell line HaCaT, as surrogates of dermal and epidermal cell lines respectively, were obtained from Eucellbank (Barcelona, Spain). Cells were cultured as previously describes (Ugartondo et al., 2009). When cells were approximately 80% confluent, they were harvested with trypsin/EDTA and seeded at a density of 8.5 x 10^4 cells/mL (3T3 in the viability assay) and 1 x 10^5 (3T3 in all the other assays, and HaCaT) in 96-well plates and then incubated for 24 h at 37 ºC and 5% CO_2. Cells between passes 8 and 28 were used in all assays.

To assess the cytotoxicity of extracts, cells were incubated overnight with concentrations of extracts ranging from 15 to 10,000 µg/mL. Cell viability was assessed by the MTT method described by Mosmann (1983). Results were given as the percentage of viability compared with control cells (the mean optical density of untreated cells was set to 100% viability).

Cytoprotection of the extracts against H_2O_2-induced damage

In the oxidative stress induction assay, cell culture medium was changed to only 5% FBS on the second day and on the third day cells were exposed to H_2O_2 concentrations ranging from 0 to 4 mM. To calculate the maximal antioxidant concentration (MAC), cells were incubated overnight with different extract concentrations never exceeding the CV_{75}, or concentration of the chemical in which the 75% of cells were viable, and then were treated for 3 hours with IC_{75} of H_2O_2, Because of the high mortality induced by the H_2O_2, the potential beneficial effects of the extract assayed will be better

evaluated. Finally, for the evaluation of the MAC efficacy, cells were incubated overnight with the MAC of each extracts and further treated with H_2O_2 concentrations ranging from 0 to 4 mM. Except for the latter, all treatments were assayed in both cell lines.

Statistical analysis

Results are expressed as mean ± S.E. of at least three independent experiments. Data were analysed by one-way analysis of variance (ANOVA) followed by Games-Howell or Dunnet's *post hoc* test for multiple comparisons between fractions with respect to the H_2O_2 controls, or the Student's *t*-tests to compare results between extracts, all using the SPSS software (SPSS Inc., Chicago, IL, USA). Statistical differences at $p<0.05$ were considered significant.

Results

Erythrocyte-based assays to study the antioxidant properties of the extracts

IC_{50} values (the concentration inhibiting 50% of the AAPH haemolytic effect) of each extract were calculated (Table 1). According to IC_{50} values, CBPE showed the highest antioxidant activity followed by SME1 and SME2, and ULE.

The antioxidant activity of the extracts was also studied by the TBARS assay on erythrocytes. TBARS (Thiobarbituric Acid Reactive Species) are used to evaluate lipid peroxidation occurring in the presence or absence of a product or a clinical condition, by the measurement of the formation of thiobarbituric acid reactive species. These chemical species react with thiobarbituric acid forming malondialdehyde, of an intense, spectrophotometrically measurable pink colour (Stocks and Dormandy 1971). In the designed experimental conditions, these assays demonstrated no antioxidant effect of the products. This result indicates that the extracts do not protect membrane lipids against the hydrogen peroxide-caused peroxidation.

Cytotoxicity of extracts in HaCaT keratinocytes and 3T3 fibroblasts

The effect of the extracts on the viability of two cell lines was assessed by the MTT assay. The results of the MTT assay are reported on Figure 1a (3T3 fibroblasts) and 1b (HaCaT keratinocytes) for those extracts with detectable toxicity at concentrations below 10,000 µg/mL. SME2 did not produce cytotoxicity at the highest concentration tested (10,000 µg/mL) on 3T3 cells; ULE showed no

cytotoxic effect neither on 3T3 cells nor on HaCaT cells (data not shown). For CBPE and SME1, a clear concentration-response relationship was established. The calculated LC_{50} values (the concentration that reduced cell viability to 50%) are shown in Table 2.

Cytoprotection of the extracts against H_2O_2-induced damage

The characterization of the antioxidant capacity of the extracts on cell lines was studied using H_2O_2 as an oxidant agent. Several studies support the use of cell lines as a sensitive model for the evaluation of oxidative stress induced by H_2O_2 (Pool-Zobel et al., 2000). Cells were exposed to different concentrations of H_2O_2 in order to establish the concentration that decreased cell viability by around 75% (IC_{75}). On 3T3 fibroblasts, a concentration of 2.2 mM of H_2O_2 was needed to observe that decrease whereas on HaCaT keratinocytes the concentration was found to be 3.85 mM, being this difference statistically significant. Overnight preincubation of cells with the extracts at concentrations ranging between 15-10,000 µg/ml, before exposure to H_2O_2 resulted in a slight modulation of cell viability. On 3T3 fibroblasts, a slight protection against H_2O_2 cytotoxicity could be attributed to the extracts (Figure 2); whereas on HaCaT keratinocytes a very slight or no protection was observed (data not shown).

Based on the results on 3T3 fibroblasts, the concentration of each extract (MAC) which conferred the highest protection against the hydrogen peroxide IC_{75} was determined for each extract and the percentage of protection are shown in Table3. Regarding the maximum percentage of protection displayed by the extracts, all of them had similar antioxidant activity with percentages of protection ranging from 6 to 10%. However, CBPE was the more potent antioxidant in terms of MAC value as it needed a lowest concentration to exert the same antioxidant effect.

As 3T3 experimented only a weak protection from hydrogen peroxide-induced oxidative stress by the extracts, the effects of MAC at lower hydrogen peroxide concentrations was tested in order to establish the antioxidant potential of the extracts at lower levels of oxidative stress. Viability of 3T3 fibroblasts was tested in presence of different hydrogen peroxide concentrations, pre-incubating the cells with the MAC concentration of each extract. The concentration-response curves (Figure 3) were used to calculate de LC_{50} of H_20_2 in the presence of the extracts as shown in Table 4. CBPE increased

significantly the LC_{50} of H_2O_2, while the other extracts had no effect (ULE) or even reduced LC_{50} (SME1, SME2).

Discussion

Addressing the green-conscious consumers, who wants active products but rejects synthetic ingredients, the potential use of natural products, either unpurified extracts or pure molecules, in cosmetics, dermopharmacy or even food industries have been extensively studied (Chen et al., 2011; Ramos et al., 2011). Different methods, or their combinations, have been proposed to study the antioxidant potential of these natural products. Out of these many possibilities, erythrocyte-based assays represent a good choice because they are quick, easy to perform, and do not need specialized equipment. Erythrocytes also have the advantage of being performed in a biological system that satisfactorily mimic a cellular environment, as the membrane structure and a substantial part of metabolism are conserved (Sadowska-Woda et al., 2010), as well as oxidative stress compensation, potentiation and/or attenuation mechanism. Extracts of algae of *Ulva* and *Sargassum* spp. genres have been previously described as antioxidants (Shalaby, 2011; Piao et al., 2011). Furthermore, the ability of *Ulva lactuca* to adsorb heavy metals has attracted industrial interest (Areco et al., 2012). Regarding the extract from *C. sativa* (CBPE), a certain antioxidant effect has been previously described (Conde, 2011a, 2011b). The results obtained by the AAPH assays indicate that the extracts have an antioxidant effect by a direct scavenging of AAPH-generated ROS. Previous research had shown the capacity of *S. muticum* extracts to scavenge radicals by the DPPH and ABTS assays and the antioxidant capacity by different non-biological tests, such as FRAP, ORAC-FL (Balboa et al. 2011). The present results confirm such property of *S. muticum,* and offers new information regarding *C. sativa* and *U. lactuca* extracts.

Antioxidant mechanisms evaluated by TBARS offers information about the anti-peroxidative effect on lipids. The extracts had no protective effect on lipid peroxidation caused by H_2O_2. TBARS and AAPH assays evaluate different antioxidant mechanisms; for this reason, some compounds may show antioxidant capacity in both assays (Inayatullah et al., 2012), whereas others show ROS scavenging capacity in the AAPH assay but no anti-peroxidative effect on lipids in the TBARS assay (Piao et al., 2011). Our results indicate that the antioxidant effect of the extracts is mediated by radical

scavenging, but not by protecting lipids from peroxidation. Other *S.muticum* extracts have been able to reduce lipid peroxidation (Hirai et al., 2011) in HaCaT keratinocytes by the DPPP method. Also, other *Ulva* species different to *U. lactuca* can inhibit lipid peroxidation (Raymundo et al., 2004). Differences in extraction and evaluation methods of the antioxidant potential of the extracts may explain the differences between SME1 and SME2 and other *S. muticum* extracts.

Cell culture assays are of greater complexity than chemical and erythrocyte-based assays. However, the antioxidant effects are evaluated from a global point of view, as the protective capacity and not a specific mechanism is evaluated. HaCaT and 3T3 cell lines are frequently combined, as models of the two main skin cell types (Tito et al., 2011). Nevertheless, immortalized cell lines may exhibit altered defence mechanisms (Nogueira et al., 2011) and such changes may affect, to a different extent, their sensitivity to oxidative insult. Although primary fibroblast are more sensitive to external aggressions than keratinocytes (Leccia et al., 1998), changes associated to the establishment of an immortalized cell line considerably hampers a similar relationship between 3T3 and HaCaT. This lack of relationship may explain the higher cytotoxicity displayed by the extracts in HaCaT keratinocytes compared to 3T3 fibroblasts, even though keratinocytes are considered more resistant to oxidative stress than fibroblasts. Moreover, HaCaT keratinocytes and 3T3 fibroblasts exhibited different sensitivity towards hydrogen peroxide insult.

This work proposes a combination of different assays, some based on erythrocytes and some on cell lines. Regarding erythrocyte-based assays, the combination of different assays evaluating different mechanisms is a major recommendation. The combination proposed in this work allows the evaluation of various antioxidant targets, thanks to the complementarity of the mechanisms studied.

It is also recommendable to perform assays in cell cultures, as some compounds can display no relevant effects in this model, despite contrary results in erythrocytes, and cell lines are one step further in extrapolation to living models. In cell line models, assaying different levels of oxidative stress may be of major interest, as weak antioxidant effects can be overwhelmed by high stress.

Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1. Concentration (µg/mL) of each extracts which showed a 50% reduction in AAPH-induced haemolysis (IC_{50}).

Extract	**IC_{50}**
SME1	181.10 ± 12.56 [a,b]
SME2	175.00 ± 6.48 [c,d]
ULE	2301.18 ± 166.47 [a,c,e]
CBPE	49.00 ± 11.13 [b,d,e]

a, b, c, d and e indicate statistically significant differences between each pair of data, in a post-hoc Games-Howell analysis ($p<0.05$).

Table 2. Concentration (µg/mL) of each product causing the death of 50% of cells (LC_{50}) in 3T3 and HaCaT cell lines, calculated from MTT results.

Extract	**LC_{50} in 3T3**	**LC_{50} in HaCaT**
SME1[a]	3321.73 ± 79.76	3592.93 ± 162.83
SME2[a]	>10,000	2309.37 ± 114.08
ULE	>10,000	>10,000
CBPE [a]	252.23 ± 15.39	169.73 ± 11.82

[a]Students's t-test statistical analysis shows statistically significant differences between LC_{50} of the product in each cell line ($p<0,05$). [b] LC_{50}s of the extracts are statistically significantly different, in a post-hoc Games-Howell analysis ($p<0,05$), except for SME2 and ULE. [c] IC_{50}s of the extracts are statistically significantly different in a post-hoc Games-Howell analysis ($p<0.05$)

Table 3. Representative values of MAC (µg/mL) for each product. In parentheses, viability increases referred to hydrogen peroxide-treated controls.

MAC (µg/mL)	**3T3 fibroblasts**
SME1	156 (6.5%)
SME2	156 (8.8%)
ULE	625 (6.0%)
CBPE	125 (10.5%)

Table 4. Concentration of hydrogen peroxide (µg/mL) causing the death of 50% of 3T3 cells (LC50), in the absence or presence of products.

Extract	**Fibroblasts 3T3**
H_2O_2	1.70 ± 0.01
SME1	1.20 ± 0.06[a]
SME2	1.33 ± 0.03[a]
ULE	1.75 ± 0.05
CBPE	2.35 ± 0.05[a]

[a]*Statistically significant differences are observed between these values and those for hydrogen-peroxide-treated controls (post-hoc bilateral Dunnet test, $p<0.05$).*

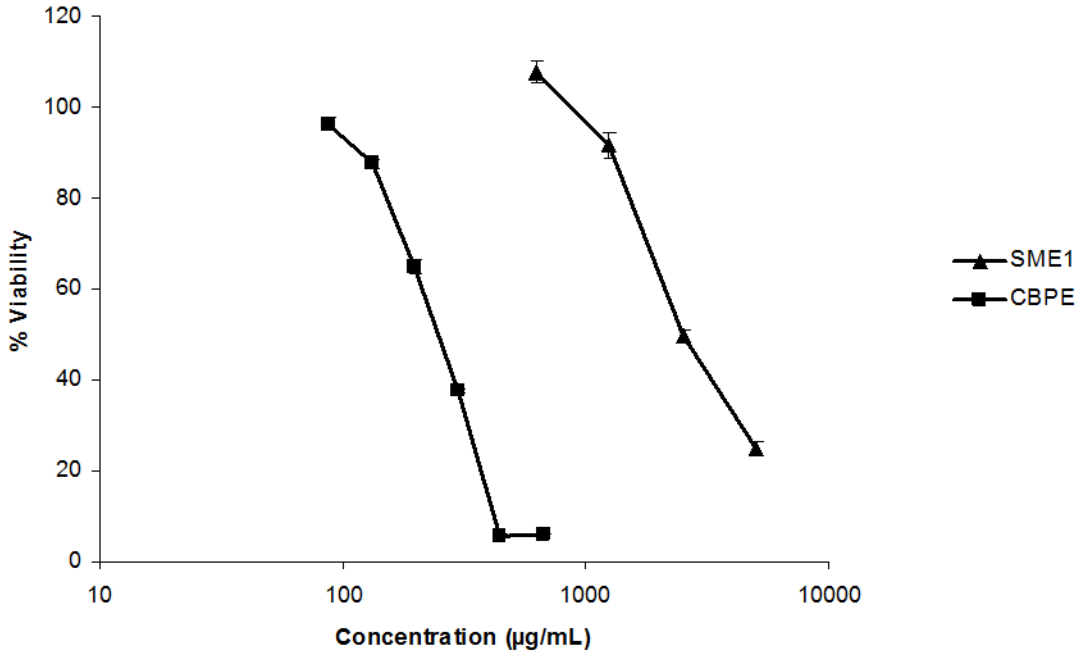
Figure captions

Figure 1. *Cell viability (expressed as a percentage from the controls) determined by the MTT assay after 24 hours exposure of the extracts in 3T3 fibroblasts (a) and in HaCaT keratinocytes (b). Mean ± SEM of at least three independent experiements*

Figure 2. *Protective effect (expressed as a percentage of viability increase) of the extracts against the oxidative stress induced by hydrogen peroxide (IC_{75} concentration) in 3T3 fibroblasts. Mean ± SEM of at least three independent experiements*

Figure 3. Viability curves relative to oxidative stress in the presence of the maximal antioxidant concentration of each extract (MAC). Mean ± SEM of at least three independent experiements

Figure 1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Concentration (µg/mL)
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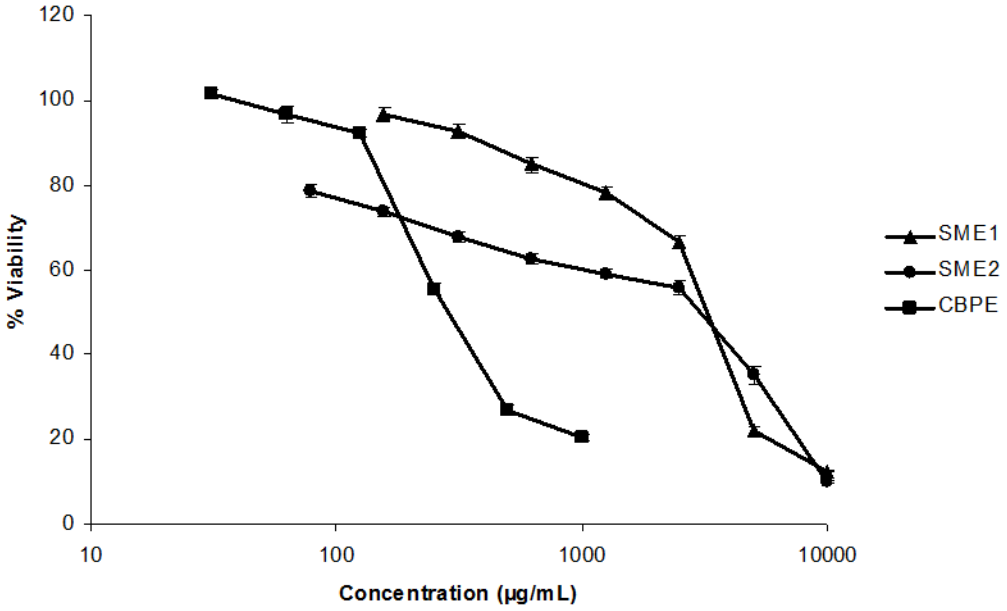
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Figure 2

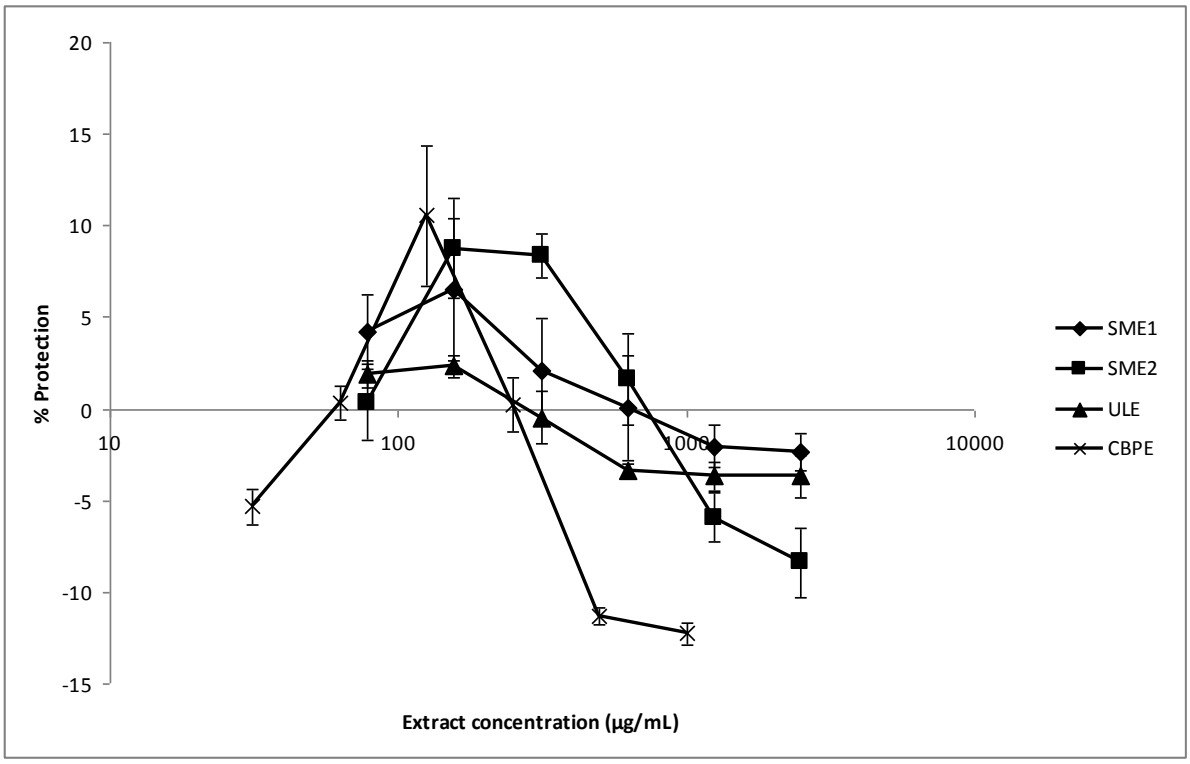
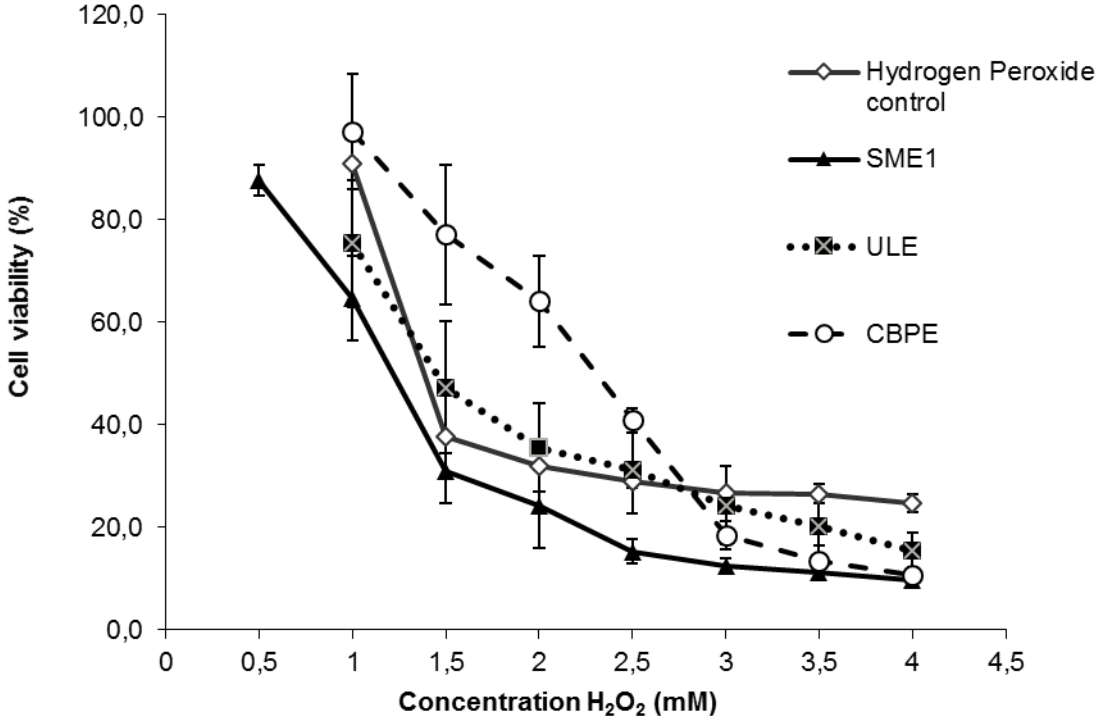

20
15
10
5
0
-5
-10
-15
% Protection
10
100
1000
10000
Extract concentration (µg/mL)
SME1
SME2
ULE
CBPE

Figure 3


120,0
100,0
80,0
60,0
40,0
20,0
0,0
Cell viability (%)
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
Concentration H_2O_2 (mM)
Hydrogen Peroxide control
SME1
ULE
CBPE