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Producing High Antioxidant Activity Extracts from Echinoderm by Products by using Pressured Liquid Extraction

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Abstract: In the present study, we attempted to produce in a laboratory scale high antioxidant activity extracts from echinoderm byproducts including green urchin (*Strongylocentrotus droebachiensis*) digestive tract and non-commercial grade gonads and Atlantic sea cucumber (*Cucumaria frondosa*) viscera by using Pressurized Liquid Extraction (PLE). We used deionised water, methanol, ethanol and isopropanol as extraction solvents. For each solvent, three extraction temperatures were tested successively, including 40, 60 and 80°C. Antioxidant activity was analysed using oxygen radical absorbance capacity assay (ORAC test). Results showed that first tests using 40°C extraction showed that ethanol and isopropanol were the best solvents to produce high antioxidant activity extracts from all the analyzed samples. When testing these two best solvents with higher temperatures (60 and 80°C) we observed ORAC values (3617-10148 μmol TE g^{-1} extract) generally better than 40°C-extracts (3463-8629 μmol TE g^{-1}) though differences were not necessary statistically significant ($p = 0.03$-0.82). Linear correlation were observed between ORAC values and sample contents in α-tocopherol ($R^2 = 0.53$), total carotenoids ($R^2 = 0.62$) and total phenols ($R^2 = 0.73$). This suggests those antioxidant potentials partly contributed to the observed ORAC responses but other compounds might be also involved. Present results showed that PLE improved antioxidant activity of echinoderm extracts up to 10 folds, compared to simple overnight stirring methods. In addition, it is observed from present results that PLE is suitable to produce high antioxidant activity echinoderm extracts, with ORAC values that might be comparable to, if not better than, some commercial antioxidant natural ingredients.

Key words: Pressurized liquid extraction, antioxidants, echinoderm by-products, organic solvents

INTRODUCTION

Natural antioxidant extracts are now subject to intensive research and national development programs. This is due to the fact that such molecules are expected to improve health conditions of human populations by reducing oxidative damage caused by pro-oxidants components. Although, most efforts have been devoted to terrestrial plants and microorganisms, little attention has been yet paid to marine invertebrates as potential sources of antioxidants (Shahidi, 2006).

Some extracts obtained from processing byproducts of green urchin (*Strongylocentrotus droebachiensis*) and Atlantic sea cucumber (*Cucumaria frondosa*) showed interesting antioxidative protection, in particular against peroxyl radicals (Mamelona *et al*., 2007, 2010). These studies showed that lipophilic fractions from the analyzed samples showed higher oxygen radical absorbance capacity (ORAC values) than their hydrophilic counterparts. When researchers conducted solid/liquid extractions with a wide range of solvents they found that antioxidant components from samples seemed to be well extracted in organic solvent of mild polarity such as acetonitrile and ethyl acetate whereas water extracts showed a much lower antioxidative protection (Mamelona *et al*., 2007, 2010). Most of ORAC values observed from these previous studies (<2000 μmol TE g^{-1} dry extract) were generally lower compared to those reported in scientific literature for natural antioxidants used in supplement formulation, with values often higher than 3000 μmol TE g^{-1} (Garrido *et al*., 2007; Monagas *et al*., 2006). Using other extraction methods might probably help to recover more antioxidant components from raw samples to final extracts.

Pressurized Liquid Extraction (PLE) is a fast and automated technique widely used in extraction of bioactive components. This technique fulfils the criteria of high yield and efficiency usually needed in this area

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(Carabias-Martinez *et al*., 2005; Ibanez *et al*., 2006). Besides, PLE allows the easy use of organic solvents, such as short-chain alcohols (C_1-C_3) that are generally recognized as safe. It makes this technique the best choice in producing ingredients for animal and human consumption. In the case of producing antioxidant extracts, PLE seems to be an appropriate choice as it has been used with success in extracting natural organic components commonly associated to antioxidative protection of biological samples, including polyphenols, ascorbic acid, α-tocopherol and carotenoid pigments (Carabias-Martinez *et al*., 2005; Ibanez *et al*., 2006).

The objective of the present study was to produce at a laboratory scale extracts with high antioxidant activity from echinoderm byproducts by using pressurized liquid extraction procedure. Extractions were carried out using deionised water and short-chain alcohols and some parameters (samples/solvent volume ratio, pressure and temperature) were changed in order to optimize the antioxidant power of resulting extracts. Antioxidant activity was measured using ORAC assays and values were compared with the well-known grape extracts that are often used in supplement formulation for human consumption.

MATERIALS AND METHODS

Chemicals and solvents: Gallic acid, α-tocopherol, β-carotene, fluorescein, Folin-Ciocalteau's phenol reagent, 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-teramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Ltd (Oakville, ON, Canada). All other chemicals and solvents were of highest commercial grade.

Sample preparation: Fresh echinoderm byproducts were obtained from local processing plants in March (sea cucumber) and August (green urchin) 2007. For sea cucumber, all the viscera, including digestive tract, gonads and respiratory tree were treated without separation and homogenized together. For green urchin, digestive tract and non-commercial grade gonads were taken apart and treated separately. Three batches of about 1 kg of fresh tissues were freeze-dried for 96 h, finely ground with commercial grinder (model Fitzmill D6 from Fitzpatrick Company, Elmhurst, IL, USA) to ~250 μm size and kept frozen (-20°C) in the dark for about 1 week. Only dry and ground samples were used for PLE procedure.

Pressurized liquid extraction procedure: Extraction was carried out using a Dionex ASE-200® extractor (Dionex Corporation, Sunnyvale, CA, USA). Stainless steel extraction cells (11 mL) and 0.22 μm cellulose filter were used. Diatomaceous earth from dionex corporation was used as a dispersing agent to enhance the contact between dry organic samples and solvents. The best sample/dispersing agent ratio to give an optimal extraction was adjusted to 1:22. This ratio gave the optimum compaction under high pressure and avoided using an additional run to complete the extraction procedure. For this purpose, 200 mg of ground sample was well mixed with 4.4 g of dispersing agent and the mixture was then compacted in the extraction cell. An average of 14 mL of liquid extract was obtained which means a weight: volume ratio between initial sample and solvent of 1/70.

Table 1: Extraction parameters used in PLE procedure using Dionex ASE-200

Extraction parameters	Unit
Temperature	40°C
Preheat	1 min
Heat	5 min
Static extraction	10 min
Pressure	1500 psi
Purge	60 sec
Flush volume	60%
Cycle	1

Four solvents of different polarity (deionised water, methanol, ethanol and isopropanol) were first tested using mild temperature (40°C). Subsequently, the two solvents giving best antioxidant extracts were tested with two higher temperatures (60 and 80°C) keeping constant the internal cell pressure. Extraction parameters that have been used with optimal results are given in Table 1. Liquid extracts were recovered in glass collectors, left a few minutes to cool down at room temperature and then stored at 4°C until analysis for antioxidant properties within 24 h after extraction to minimize possible degradation of sensitive products. The dry mass of each extract was determined after evaporation to dryness of an aliquot of the extract using either a continuous nitrogen flow with solvents or freeze-drying method with water.

ORAC assays: The analytical procedure has been adapted from a method published by Ou *et al.* (2001), using 96-well microplates and a SpectraMaxGemini spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA). After appropriate dilution, a 30 μL extract aliquot was added to each well in which 150 μL of 1.04 μM fluorescein and 30 μL of 162 mM 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) were previously added. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analog of α-tocopherol, was used as positive control and phosphate buffer pH 7.4 was used as the analytical blank. The reactive mixture was incubated at 37.5°C for 90 min. Final results were calculated as a function of the

difference between the surface under the curves between blanks and extracts and the antioxidant properties expressed as ORAC values in equivalent micromolar of Trolox by dry weight of the sample (μmol TE g^{-1} extract).

Analysis of antioxidant potential compounds: Only extracts obtained from the 60°C extraction protocol were used for antioxidant compound analysis as it was an efficient one with a good preservation of the final products. Extracts obtained at 80°C showed chemical instability as evidenced by whitish residues appearing in the bottom of the glass collectors after only 24 h storage in the refrigerator (4°C) or at room temperature (22°C). This indicated that some components extracted at elevated temperature precipitated out whereas this was not observed after three months with lower temperature extracts.

Extracts were analysed for their contents in α-tocopherol, total phenols and carotenoids. Determination of α-tocopherol was carried out using slightly modified silylation derivatization method following Cunha *et al.* (2006). Hexane extract containing tocopherol family was obtained after phase separation using hexane/methanol mixture and deionised water. The hexane phase was separated; an aliquot of 1 mL was evaporated to dryness and then submitted to silylation using N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide. The derivatized extract was injected in splitless mode in a gas chromatograph (Varian CP 3800), coupled to a mass spectrometer (Varian Saturn 2200; Varian Inc., Palo Alto, CA, USA) for separation, identification and quantification of α-tocopherol. Separation of compounds was carried out using a capillary non-polar column, Simplicity-1®, 0.25 mm ID×30 m, 0.25 μm coating thickness.

Total carotenoids were analysed using spectrophotometric method following Symonds *et al.* (2007). Dried extracts were dissolved in acetone, after appropriate dilution absorbance of orange coloured solution was measured at 447 nm (Spectra-FluorPlus, Tecan, Durham, NC, USA), quantification was conducted using standard solutions of β-carotene ranging from 2-200 mg g^{-1} (Symonds *et al.*, 2007). The extinction coefficient, $E_{(1\%, 1\,cm)}$ = 2500, was used to calculate the total carotenoid content. Variability between replicates was <5% for all analyzed extracts.

Total phenols were analysed in dried extracts using folin-ciocalteau method following protocols previously described by Mamelona *et al.* (2007). Samples were first dissolved in methanol. After appropriate dilution with deionised water solution was mixed with folin ciocalteau reagent. Absorbance of the blue coloured resulting mixture was measured at 750 nm against blank; quantification was carried out using standard solutions of gallic acid between 200 and 2000 μg g^{-1}. Obtained standard curves showed linear relationship (R^2 = 0.99).

Statistical analysis: All procedures were carried at least in triplicate. Statistical treatments were performed using Sigma Stat® (Jandel Scientific Software, San Rafael, CA, USA) at 5% significance error level. Comparisons between groups were performed using parametric (t-test, one-way ANOVA) and non-parametric (Mann-Whitney Rank Sum test) tests. When differences were detected we performed post hoc comparisons using the test of Student Newman Keuls (SNK) or non-parametric tests. Relationship between ORAC values and antioxidant potential components were determined using pearson regression.

RESULTS AND DISCUSSION

The first extraction tests using 40°C temperature gave extracts showing highly variable ORAC values ranging from 54 μmol TE g^{-1} for water to 8630 μmol TE g^{-1} for isopropanol (Fig. 1). The effect of solvent was clearly

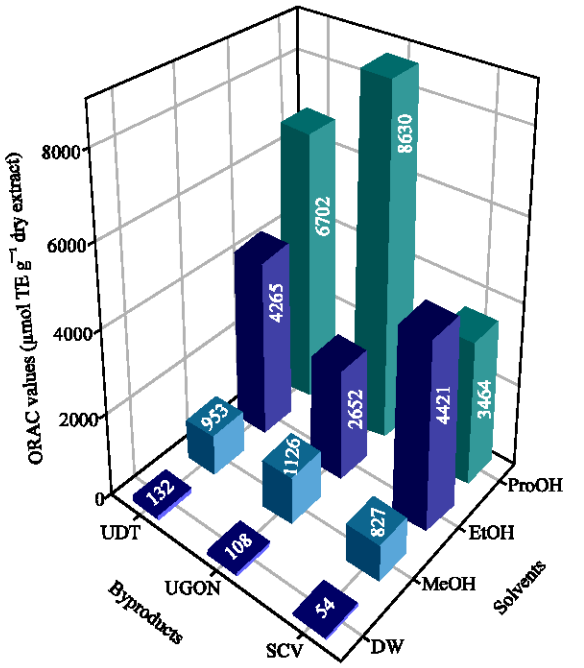

Fig. 1: ORAC values of extracts obtained at 40°C using deionized water, methanol, ethanol and isopropanol. Data are expressed as micromoles of Trolox equivalents g^{-1} of dry extract. All assays were conducted in triplicate and mean values are used. UDT: Urchin digestive tract; UGON: Urchin gonads; SCV: Sea cucumber viscera; DW: Deionized water; MeOH: Methanol; EtOH: Ethanol, ProOH: Isopropanol

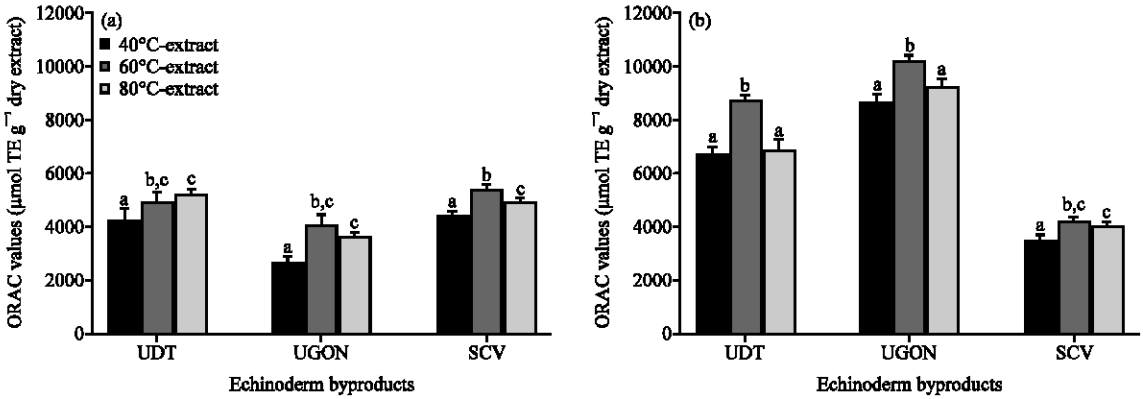

Fig. 2: ORAC values of extracts obtained at 40, 60 and 80°C using (a) ethanol and (b) isopropanol. Data are expressed as micromoles of Trolox equivalents g^{-1} of dry extract. All assays were conducted in triplicate and mean values are used. The vertical bars represent the standard deviation of each data point. Means within each group with different letters (a-c) differ significantly ($p<0.05$) from each others. UDT: Urchin digestive tract; UGON: Urchin gonads; SCV: Sea cucumber viscera

Table 2: Contents of 60°C ethanol and isopropanol extracts in α-tocopherol, total carotenoids and phenols

Antioxidant potential compounds	Urchin digestive tract		Urchin gonads		Sea cucumber viscera	
	Ethanol	Isopropanol	Ethanol	Isopropanol	Ethanol	Isopropanol
α-tocopherol (µg g^{-1})	310	703	377	229	220	145
Total carotenoids (mg g^{-1})	80	114	75	70	60	33
Total phenols (µg g^{-1})	1051	965	794	1246	894	464

Values are means from triplicate determination

observed for all extracted tissues as ORAC values showed a general increase of ORAC values following the solvent series: water<methanol<ethanol≤isopropanol. However, a single exception was observed for sea cucumber viscera where ethanol extract exhibited an antioxidant power greater than isopropanol extract ($p<0.05$). The best ORAC values, obtained from ethanol and isopropanol extracts, were far higher (2652 to 8630 µmol TE g^{-1}) than those observed in previous studies, obtained from a simple stirring at room temperature (20°C) of raw samples using solvents of about the same extraction power (140 to 2100 µmol TE g^{-1}) (Mamelona *et al*., 2007, 2010; Zhong *et al*., 2007). We first reports that PLE is a suitable technique to improve antioxidant activity of echinoderm extracts, notably when ethanol and isopropanol are used and temperature increased.

When testing extraction efficiency of ethanol and isopropanol with higher temperatures (60 and 80°C) we found ORAC values generally better than extracts obtained at 40°C although differences were not necessarily significant ($p = 0.03$-0.82). The degree of improvement slightly changed as a function of extracted tissue and solvent, varying from 15 to 53%. The extracts obtained by using 60°C temperature provided ORAC values generally better than those obtained by using 80°C, with the exception found in ethanol extracts obtained from urchin digestive tract (Fig. 2a, b). Present results come in support to Ju and Howard (2003) that observed the effect of extraction temperature on antioxidant properties of the obtained extracts when using PLE procedure. As for the 40°C extraction, isopropanol extracts obtained at 60 and 80°C showed generally higher ORAC values compared to those obtained with ethanol at the same temperatures.

Concentrations of α-tocopherol, carotenoids and total phenols in ethanol and isopropanol 60°C-extracts are given in Table 2. The ethanol extracts showed much better richness in these three potent antioxidants compared to isopropanol, with some particular cases being observed. In fact, isopropanol extracts from green urchin digestive tract and gonads were higher compared to ethanol extracts in α-tocopherol and total phenol contents, respectively. A correlation study between ORAC values of extracts and their potential antioxidant contents indicated that each of them were partly responsible for the ORAC response of extracts with linear correlation coefficients (R^2) ranging from 0.53 for α-tocopherol to 0.73 for total phenols (Fig. 3a-c). This suggests a combined action of these compounds in the response of the ORAC assay although other active substances are most probably also involved. This comes in support to previous studies showing antioxidant effects of these three natural components (Mamelona *et al*., 2007;

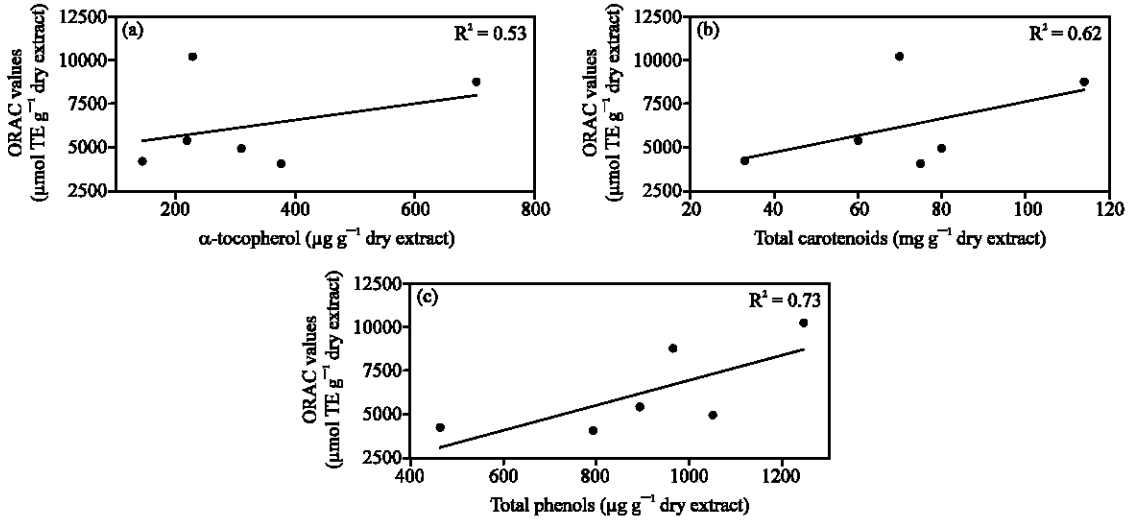

Fig. 3: Relationship between ORAC values for extracts at 60°C and their contents in (a) α-tocopherol, (b) total carotenoids and (c) total phenols. Solid lines represent linear regression curves

Table 3: Comparison of ORAC values of ethanol and isopropanol extracts obtained at 60°C to some commercial dietary ingredients used in formulation of supplements

Ingredient	Source	Extraction method	ORAC value[a]	References
Echinoderm byproducts No. 1	Green urchin or atlantic sea cucumber viscera	PLE extraction using ethanol	4061-5390[b]	This study
Echinoderm byproducts No. 2	Green urchin or atlantic sea cucumber viscera	PLE extraction using isopropanol	4200-10148[b]	
Red wine marc extract	Local markets in Madrid, Spain	NR	3480[c]	Garrido *et al.* (2007)
Grape seed extract	Local markets in Madrid, Spain	NR	8260[c]	
Fresh grape skins	Skins of *Vitis vinifera* L. grape	Water extraction	2230-4300[d]	Monagas *et al.* (2006)
Fresh grape skins	Skins of *Vitis vinifera* L. grape	Hydro-alcoholic extraction	5490-6320[d]	
Grape leaves	Leaves of *Vitis vinifera* L. grape	Alcoholic extraction	2190[c]	

[a]Expressed as µmol TE g^{-1} of extract, values come from triplicate determination; NR: Not reported; [b]Range for all extracts are given; [c]Values from determination on only one batch, expressed as Total-ORAC (H-ORAC + L-ORAC); [d]Values from determination on five different batches

Monagas *et al.*, 2005; Seppanen *et al.*, 2010; Woodall *et al.*, 1997; Zheng and Wang, 2003).

Natural extracts from grapes or winery factories are often used in formulation of functional foods intended to promise antioxidative protection (Schieber *et al.*, 2001; Monagas *et al.*, 2005). From our results we observed that PLE might be a useful method to recover much more powerful antioxidants from urchin tissues. It allowed to improve ORAC values of the obtained extracts close to if not better than extracts from grapes and winery factories. As shown in Table 3, ethanol and isopropanol 60°C-extracts obtained from the present study exhibited better antioxidative protection than most antioxidant extracts from grapes. It should also be noted that isopropanol extracts from green urchin non-commercial gonads showed particularly high ORAC values (~10,000 µmol TE g^{-1}) compared to grape extracts (not more than 8,000 µmol TE g^{-1}) (Garrido *et al.*, 2007; Monagas *et al.*, 2006).

CONCLUSION

Pressurized liquid extraction procedure allowed an improvement of antioxidant activity of echinoderm extracts at a laboratory scale, notably when using ethanol and isopropanol as solvents. Extraction temperature affected both antioxidative protection efficiency and chemical stability of the resulting extracts, with 60°C as the best choice. Common natural antioxidants such as α-tocopherol, carotenoids and phenolic compounds appeared to be responsible of the observed antioxidative protection but the contribution of unidentified antioxidants is also suggested. Further studies using guided assay are needed to analyze those compounds.

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