

Article

Topical Benefits of Two Fucoïdan-Rich Extracts from Marine Macroalgae

J. Helen Fitton ^{1,*}, Giorgio Dell'Acqua ², Vicki-Anne Gardiner ¹, Samuel S. Karpiniec ¹, Damien N. Stringer ¹ and Emma Davis ¹

¹ Marinova Pty Ltd., 249 Kennedy Drive, Cambridge, Tasmania 7170, Australia; E-Mails: info@marinova.com.au (V.-A.G.); sam.karpiniec@marinova.com.au (S.S.K.); damien.stringer@marinova.com.au (D.N.S.); emma.davis@marinova.com.au (E.D.)

² Dellacqua Consulting LLC, Jersey City, NJ 07302, USA; E-Mail: giorgio_dellacqua@hotmail.com

* Author to whom correspondence should be addressed; E-Mail: helen.fitton@marinova.com.au; Tel.: +61-3-6248-5800; Fax: +61-3-6248-4062.

Academic Editor: Enzo Berardesca

Received: 4 March 2015 / Accepted: 2 April 2015 / Published: 16 April 2015

Abstract: Two concentrated and well-characterized fucoïdan-rich extracts were investigated to determine their benefits in topical applications. An *Undaria pinnatifida* extract, containing 85% fucoïdan, and a *Fucus vesiculosus* co-extract, containing 60% fucoïdan and 30% polyphenol, were assessed in a number of *in vitro* assays to measure the effect of the extracts on enzyme inhibition, glycation, antioxidant activity and Sirtuin 1 (*SIRT1*) protein expression. Double-blind, placebo-controlled clinical studies were also conducted to measure soothing, protection, wrinkle depth, brightness and skin spot intensity. Both extracts demonstrated marked inhibitory effects on processes linked to skin aging, including the increased expression of *SIRT1 in vitro*. Clinical testing established the efficacy of the extracts in a range of the tested applications, relative to placebo. The *Fucus vesiculosus* extract with high polyphenol content demonstrated additional *in vitro* antioxidant activity, as well as improved efficacy in skin brightening applications, relative to placebo. The major effects of the *Undaria pinnatifida* extract aided skin immunity, soothing and protection, while the *Fucus vesiculosus* extract most significantly affected age spot reduction and increased brightness, soothing and protection.

Keywords: fucoidan; cell culture; skin physiology/structure; genetic analysis; polyphloroglucinol; superoxide; brightness; age spots

1. Introduction

Marine macroalgae contain a variety of polysaccharides for both structural and protective functions. The polysaccharide, “fucoidan”, and the marine polyphenols, “polyphloroglucinols”, are molecules produced by macroalgae in order to protect themselves from high UV conditions, from predation and against attack by marine pathogens. Fucoidans are fucose-rich sulfated polysaccharides found only in brown macroalgae (Phaeophyceae) and echinoderms. These polysaccharides differ in their sugar backbone composition, structure and sulfation patterns according to the species of origin and the extraction techniques used. *Undaria pinnatifida* yields a fucoidan with a high proportion of galactose in the backbone that, in addition to sulfate groups, is also extensively acetylated. *Fucus vesiculosus* yields a fucoidan with a predominance of fucose in the sugar backbone, and little acetylation. Both fucoidans are highly branched and heterodisperse.

In previous studies, fucoidans have displayed bioactivity in several areas [1]. They have a heparin-like anticoagulant activity, inhibit selectin function, inhibit viral entry to cells, and appear to be immunomodulatory. Fucoidans from different species have been shown to inhibit Ultra-violet (UV)-induced matrix metalloprotease (MMP) expression *in vitro* [2–4]. Senni showed that fucoidans are extensive inhibitors of matrix metalloproteases, while fucoidan (or fucoidan-containing) extracts from *Fucus vesiculosus* and *Ascophyllum nodosum* have been shown to inhibit elastase [5,6]. Topical *Undaria pinnatifida* fucoidan was as effective as a topical steroid cream when applied to atopic dermatitis in a mouse model [7], and while the mechanism for this effect is uncertain, the result demonstrates that fucoidan is clearly promising as a therapeutic target, especially given the side effects of longer term topical steroid use.

Polyphloroglucinols have been shown to have a marked protective effect on UV damage models *in vivo* [8]. Polyphenols in general are also tyrosinase [9] and elastase [6] inhibitory substances and are known to have antioxidant [10] effects. Free radicals in the skin—generated by sun exposure, chemical exposure and normal skin aging processes—have been shown to strongly affect skin aging [11]. As such, topical inhibitors of free radical action show promise as cosmetic ingredients.

Extracts from marine macroalgae often comprise “whole plant extracts” with unknown quantities of active ingredients, which can thus make identifying the cause of their functionality a difficult task. In this paper we used *in vitro* and clinical studies to investigate two specific, well-characterized macroalgal extracts—with high concentrations of fucoidan and marine polyphenols—to assess their efficacy as topical cosmetic ingredients. These concentrated extracts are derived from the annual macroalgae *Undaria pinnatifida* (*Undaria pinnatifida* extract, containing 85% fucoidan), and from *Fucus vesiculosus* (*Fucus vesiculosus* extract, containing a co-extract of 60% fucoidan and 30% polyphloroglucinol).

2. Experimental Section

The two macroalgal extracts used in the experiments described in this paper were produced by Marinova Pty Ltd. (Tasmania, Australia), and specific properties are described in Table 1. Both products were assessed for phototoxicity ($n = 50$) and photoallergenicity ($n = 10$) in human clinical studies. Neither product was a primary sensitizer or phototoxic (see Supplementary Materials 1).

Table 1. Description of *Fucus vesiculosus* extract and *Undaria pinnatifida* extract.

Fucoidan Extract	Neutral Carbohydrates	Sulfate	Cations (approx.)	Fucoidan	Polyphenol
<i>Fucus vesiculosus</i> Extract	43.7%	10.1%	3%	58.6%	33.7%
<i>Undaria pinnatifida</i> Extract	48.8%	27.4%	9%	89.6%	<2%

2.1. Enzyme Inhibition Assays

2.1.1. Elastase

Human neutrophil elastase inhibitory activity was assessed using the method of Lee [12]. Enzyme activity was measured using [*N*-Succ-(Ala)₃-*p*-nitroanilide] as the substrate, and the release of *p*-nitroaniline was monitored spectrophotometrically at 410 nm, in the presence of macroalgal extracts.

2.1.2. Collagenase

Collagenase inhibitory activity was assessed using a fluorescein-conjugated collagen type 1 substrate. Bacterial collagenase (Sigma-Aldrich, St. Louis, MO, USA) was used to digest the substrate, and the resulting fluorescent signal was used to assess the rate of the reaction in the presence of inhibitory extracts.

2.1.3. Tyrosinase

Mushroom tyrosinase inhibitory activity was assessed in the 5,6-Dioxo-2,3,5,6-tetrahydro-1H-indole-2-carboxylic acid dopachrome assay, according to methods described by Fling [13] and as modified by Kang [9]. In brief, L-3,4-dihydroxyphenylalanine (L-DOPA) was used as a substrate for tyrosinase. The dopachrome-colored reaction product was then quantified spectrophotometrically at 550 nm.

2.2. Glycation Inhibition Assay

The glycation inhibition assay is based on a modification of the methods of Kiho [14] and Choi [15]. Bovine serum albumin, fructose and either a standard inhibitor of glycation, or the macroalgal extracts were used. The positive standard inhibitor was amino-guanidine. Glass vials in triplicate were sealed and incubated for seven days in the dark at 37 °C. Autofluorescence of the glycated protein was measured on a plate reader (excitation 370 nm, emission 440 nm).

2.3. Free Radical Inhibition: The ORAC5.0 Test

Oxygen Radical Absorbance Capacity (ORAC) assays are acknowledged methods that measure antioxidant scavenging activity against oxygen radicals that are known to be involved in the pathogenesis of aging and many common diseases. ORAC5.0 consists of five types of ORAC assays that evaluate the antioxidant capacity of a material against five primary reactive oxygen species (ROs, commonly called “oxygen radicals”) found in humans: peroxy radical, hydroxyl radical, superoxide anion, singlet oxygen, and peroxyxynitrite. In brief, a fluorescent signal decay is monitored after exposure to the radicals. Peroxyl radicals are generated by Azobis(2-amidinopropane) dihydrochloride, hydroethidine was used as a probe in measuring superoxide scavenging capacity and also to measure the singlet oxygen. Peroxyxynitrite activity was measured by oxidation of the dye dihydrorhodamine 123. These methods are described by Mullen *et al.* [16]. *Fucus vesiculosus* extract was assessed in this assay. Trolox, a water-soluble form of vitamin E, was used as the reference standard, and the results are expressed as μmole Trolox equivalency per gram (or milliliter) of extract.

2.4. UV Absorbance Range

Both extracts were assessed for UV absorbance from 200 to 800 nm at a concentration of 0.1% w/v in water, using an HP8452A spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA). This allowed for assessment of the UV-absorbance properties of each compound over the UV ranges important to human health: UVC (100–280 nm), UVB (280–315 nm), and UVA (315–400 nm).

2.5. Sirtuin 1 (SIRT1) Protein Expression

MiaPaCa-2 cells (human pancreas epithelial cell line, ATCC# CRL-1420) were pre-treated with or without the presence of testing samples. After treatment, the lysed cells were assayed using antibodies for human Sirtuin 1 (*SIRT1*) protein, with a horseradish peroxidase (HRP)-conjugated secondary detector antibody and 3,3',5,5'-tetramethylbenzidine (TMB) substrate to react with bound HRP. The expression level of *SIRT1* in cell lysate was then monitored via optical investigation of the reaction mix. *SIRT1* expression levels of human cells treated with and without test materials were compared, and the maximum percentage of *SIRT1* expression change was reported. The concentration that induced the maximum percentage of the *SIRT1* expression change was also noted [17].

2.6. Gene Expression in Skin Models

Ten-day-old reconstructed human epidermis was topically treated (or not) with the test compound and incubated for 24 h. In this method, normal human keratinocytes are cultured at high cell density in serum-free and high calcium (1.5 mM) media on an inert polycarbonate filter at the air–liquid interface. The stratified cultures are histologically similar to those observed *in vivo* in the epidermis [18]. Each sample was replicated ($n = 2$). After incubation, cells were retreated with the test compound and irradiated with UVB at 500 mJ/cm^2 , +UVA at 6.98 J/cm^2 , using a SOL500 sun simulator equipped with an H2 filter (Dr. Hönle AG, München, Germany). After irradiation, the cells were topically retreated with the test compound and incubated for 4 or 24 h. At the end of the incubation, all cells were washed in phosphate-buffered saline and frozen at $-80 \text{ }^\circ\text{C}$.

The modulation of gene expression related to inflammation was evaluated using a UV irradiated reconstructed human epidermis. After extraction of RNA, complementary DNA was synthesized using reverse transcriptase. RT-qPCR extracted mRNA was analyzed on a dedicated PCR array (mQPA-INFLAMM-TISSUE-64) containing 64 target genes including three house-keeping genes. The threshold for stimulation to be determined is >150% of control (slight stimulation), >200% (stimulation), >300% (strong stimulation). Similarly, inhibition was determined at >30% (inhibition) or >50% (strong inhibition). *Undaria pinnatifida* extract was assessed in this assay at concentrations of 30 and 100 µg/mL.

2.7. Clinical Test Protection and Soothing

Twenty-five Caucasian subjects participated in a placebo-controlled study to assess the ability of a 0.3% w/v gel formulation (see Supplementary Materials 2 for formulation) to protect from and soothe damage caused by UV irradiation from a solar simulator (Farcoderm, San Martino Siccomario, Italy) at 1.25× the minimal erythemal dose (MED). The MED (the lowest energy quantity of UVA + UVB radiation causing a slight but well-defined erythema) was previously evaluated for each volunteer. Damage was assessed by measuring erythema and transepithelial water loss (TEWL) at various time points after irradiation. The evaluation of erythema was performed by a MEXAMETER® MX 18 (Courage + Khazaka electronic GmbH, Köln, Germany). TEWL was assessed by a Tewameter 300R (Courage + Khazaka electronic GmbH). Formulations containing extracts or placebo were applied either before and after, or only after, exposure to a UV source according to several defined protocols (Table 2). The quantity of the formulation applied by hand on the skin site was equal to 2 mg/cm². The protective effect was calculated as the decrease of the damage (skin erythema or TEWL) induced by UV exposure (vs. control area, untreated). The soothing effect was calculated as the decrease of the damage (skin erythema or TEWL) induced by UV exposure (vs. the respective T0). The study was carried out under a dermatologist's supervision, in agreement with the ethical principles for medical research (Helsinki Declaration and successive amendments).

Table 2. Study schemes and sampling schedules for clinical protection and soothing.

Study Scheme 1	Study Scheme 2	Study Scheme 3
Pre-treatment (30 min before UV exposure) + UV exposure (1.25× MED) + Treatment 20 ± 4 h after UV exposure	Pre-treatment (30 min before UV exposure) + UV exposure (1.25× MED) + Treatment soon after UV exposure	UV exposure (1.25× MED) + Treatment 20 ± 4 h after UV exposure
Sampling Schedule 1	Sampling Schedule 2	Sampling Schedule 3
T(-1): before pre-treatment, normal skin	T(-1): before the pre-treatment, normal skin	T(-1): before UV exposure, normal skin
T(-1, pr): 30 min after pre-treatment before UV exposure	T(-1, pr): 30 min after pre-treatment before UV exposure	–
T0: 20 ± 4 h after UV exposure	T24h: 24 h after both product application and UV exposure	T0: 20 ± 4 h after UV exposure
T1h: 1 h after product application	–	T1h: 1 h after product application
T2h: 2 h after product application	–	T2h: 2 h after product application
T24h: 24 h after product application	T48h: 48 h after product application and UV exposure	T24h: 24 h after product application

2.8. Clinical Test on *Fucus vesiculosus* Extract for Age Spots, Brightness and Wrinkles

A double-blind placebo-controlled hemi-face study was carried out over 60 days on twenty Caucasian subjects using a 0.3% w/v cream formulation containing *Fucus vesiculosus* extract and a placebo formulation (see Supplementary Materials 2 for formulation). The subjects were instructed to apply the preparations in the morning and at night, on perfectly cleaned face skin, applying “product A” to a hemi-face and “product B” to the contralateral one following the study randomization scheme. Participants were also instructed to clean hands before each product application.

Subjects were assessed at 15, 30 and 60 days for brightness (gloss), wrinkle depth, and the melanin index of an age spot. Non-invasive instrumentation was used by a trained operator to assess skin parameters on each side of the face on each visit. To assess brightness, gloss value was evaluated by means of a CM-700d spectrophotometer/colorimeter (Konica Minolta, Tokyo, Japan). Eye contour wrinkles were quantitatively assessed by Primos 3D (GFMesstechnik GmbH, Teltow, Germany). The evaluation of melanin index on skin spots was performed by a MEXAMETER[®] MX 18 (Courage + Khazaka electronic GmbH) probe. In addition, a dermatologist assessed the subjects at each visit, and scored the appearance of the skin with regard to each parameter. Subject cream application schedules were randomized using Efron’s biased coins method. A normality test (Shapiro Wilk) was conducted on all the data. Once the normality of data was accepted, the inter-group comparisons were performed by Student’s *t*-test.

3. Results and Discussion

3.1. Enzyme Inhibition Assay

3.1.1. Elastase

Both *Fucus vesiculosus* extract and *Undaria pinnatifida* extract were effective elastase inhibitors at half-maximal inhibitory concentrations of less than 100 µg/mL (Table 3).

3.1.2. Collagenase

Both *Fucus vesiculosus* extract and *Undaria pinnatifida* extract were effective collagenase inhibitors as illustrated in Table 3.

3.1.3. Tyrosinase

The *Fucus vesiculosus* extract was a highly effective inhibitor of tyrosinase at a half-maximal inhibitory concentration of less than 50 µg/mL, and was also significantly more active than the *Undaria pinnatifida* extract, as illustrated in Table 3.

Table 3. Extract elastase, tyrosinase and collagenase inhibition results (percent inhibition).

Extract	Elastase Inhibition	Elastase Inhibition EC ₅₀	Tyrosinase Inhibition	Tyrosinase Inhibition EC ₅₀	Collagenase	Collagenase IC ₅₀
<i>Fucus vesiculosus</i> extract	99% at 0.1 mg/mL	76 µg/mL	99% at 0.02 mg/mL	33 µg/mL	99% at 0.1 mg/mL	60 µg/mL
<i>Undaria pinnatifida</i> extract	99% at 0.1 mg/mL	68 µg/mL	5% at 1 mg/mL	n/a	99% at 0.1 mg/mL	55 µg/mL

3.2. Glycation Inhibition Assay

Both the *Fucus vesiculosus* and *Undaria pinnatifida* extracts were effective inhibitors of glycation as illustrated in Table 4.

Table 4. Extract glycation inhibition results (percent inhibition).

Compound	Concentration	Glycation Inhibition
<i>Fucus vesiculosus</i> extract	0.1 mg/mL	31%
	0.2 mg/mL	45%
<i>Undaria pinnatifida</i> extract	0.1 mg/mL	33%
	0.2 mg/mL	50%
Aminoguanidine	0.1 mg/mL	24%
	0.2 mg/mL	50%

3.3. Free Radical Inhibition: The ORAC5.0 Test

The ORAC5.0 assay results demonstrated the high free radical inhibition capability of the *Fucus vesiculosus* extract—with a particularly high antioxidant power noted against superoxide anion—as illustrated in Table 5. Antioxidant capacity is expressed in terms of Trolox Equivalents (TE) per gram.

Table 5. ORAC5.0 assay results for *Fucus vesiculosus* extract.

Free Radical Type	Antioxidant Power Result (µmol TE/g)
Peroxyl	1,144
Hydroxyl	1,955
Peroxynitrite	138
Superoxide	23,025
Singlet oxygen	925
Total	27,187

3.4. UV Absorbance Range

The obtained spectra (Figure 1) showed the *Fucus vesiculosus* extract to exhibit a more superior absorptivity than the *Undaria pinnatifida* extract, and over a greater range of wavelengths extending into the UVB and UVA regions.

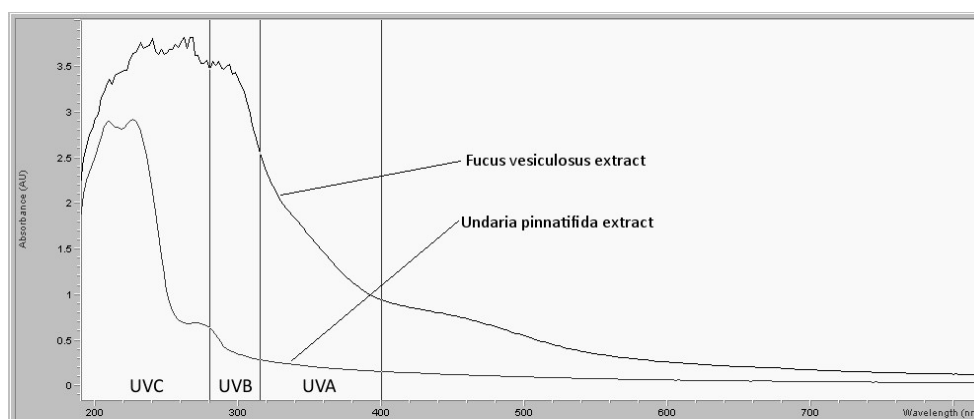


Figure 1. Ultraviolet (UV)-Vis absorbance spectra of macroalgal extracts at 0.1% w/v.

3.5. SIRT1 Protein Expression

The maximum increase in *SIRT1* expression and the effective concentrations at which this was achieved are noted in Table 6. Both extracts increased the expression of the *SIRT1* protein *in vitro*, with the *Fucus vesiculosus* extract demonstrating the greatest expression change.

Table 6. Maximum increase in *SIRT1* expression (percent change).

Extract	Maximum SIRT1 Expression Change	Effective Concentration
<i>Fucus vesiculosus</i>	32.4%	4.9 µg/mL
<i>Undaria pinnatifida</i>	28.8%	14.8 µg/mL

3.6. Gene Expression in a Skin Substitute

The results are summarized in Table 7. Genes for Toll-like receptors 2 and 3 were strongly activated, at 387% and 229% respectively.

There was a large increase in the expression of wound-healing genes for the main matrix metalloproteases: at four hours, gene expression in skin treated with the *Undaria pinnatifida* extract was more than doubled as compared to the control, showing an enhanced wound-healing signal. One enzyme gene was inhibited (MMP14).

Table 7. QPA-INFLAM-TiSSUE-64 model. Summary of *Undaria pinnatifida* extract on gene expression profile of UVB and UVA irradiated reconstructed human epidermis. Irradiation at 500 mJ/cm². (percent change *versus* irradiated control, after 4 h of exposure).

Category	Genes	30 µg/mL	100 µg/mL
Antimicrobial peptide, innate immunity	TLR2	134	387
	TLR3	99	229
Inflammatory cytokine	IL6	<50	<50
Extra cellular matrix enzymes	MMP1	157	202
	MMP3	151	233
	MMP9	183	226
	MMP14	27	31
	VEGFA	50	71

3.7. Clinical Test Protection and Soothing

Both extracts were proven to be clinically effective soothing and protecting agents with significant reduction in erythema and TEWL as compared to placebo and control. In Study Scheme 1, the products were applied before UV exposure and then again, after 24 h (Table 2). The relative protective and soothing effects are expressed in Tables 8 and 9 for cream preparations of the *Fucus vesiculosus* and *Undaria pinnatifida* extracts. Out of the three study schemes, this first scheme of application generated the most protection; however, the other two schemes (application before and soon after exposure, or only after exposure) also markedly decreased TEWL and erythema.

Table 8. Transepithelial water loss after application of placebo or active cream, as compared to control (Study Scheme 1); $n = 25$ (percent change of mean vs. control).

Product	T0 (%)	T1h (%)	T2h (%)	T24h (%)
<i>Fucus vesiculosus</i> extract	-7.9	-21.9	-22.1	-18.3
<i>Undaria pinnatifida</i> extract	-7.3	-19.8	-19.6	-17.1
Placebo	-1.6	-8.0	-7.6	-2.6

Table 9. Erythema after application of placebo or active cream, as compared to control (Study Scheme 1); $n = 25$ (percent change of mean vs. control).

Product	T0 (%)	T1h (%)	T2h (%)	T24h (%)
<i>Fucus vesiculosus</i> extract	-10.6	-21.6	-22.6	-14.7
<i>Undaria pinnatifida</i> extract	-9.0	-18.5	-19.6	-12.1
Placebo	-1.4	-2.2	-2.0	-0.2

Statistical analysis was performed by way of t -testing for paired samples, to compare both *Undaria pinnatifida* and *Fucus vesiculosus* extracts to the placebo and control data sets in Study Scheme 1, for both TEWL and erythema. Significant differences were found between the fucoidan extracts and the placebo against control tests in all cases, as summarized in Table 10.

Table 10. Significant differences between data sets for Study Scheme 1 (Student's t -test for paired data).

Extract	TEWL %		Erythema %	
	Control	Placebo	Control	Placebo
<i>Fucus vesiculosus</i> extract	$p < 0.001$	$p < 0.002$	$p < 0.001$	$p < 0.001$
<i>Undaria pinnatifida</i> extract	$p < 0.001$	$p < 0.002$	$p < 0.001$	$p < 0.001$

The data from Study Schemes 2 and 3 showed that both extracts were protective and soothing as summarized in Tables 11 and 12, respectively. These observations were statistically significant with regard to both placebo and control (t -test for paired data, $p < 0.05$). The relative protective and soothing effects on erythema and TEWL were slightly greater for the *Fucus vesiculosus* extract than for the *Undaria pinnatifida* extract; however, both extracts demonstrated significant protective and soothing effects.

Table 11. Erythema and TEWL Study Scheme 2, as compared to control ($n = 25$) (percent change of mean vs. control).

Extract	TEWL %		Erythema %	
	24 h	48 h	24 h	48 h
<i>Fucus vesiculosus</i>	-8.4	-16.6	-10.4	-8.9
<i>Undaria pinnatifida</i>	-7.7	-14.4	-10.3	-8.1
Placebo	-1.2	-2.3	-1.2	-2.5

Table 12. Erythema and TEWL Study Scheme 3, as compared to control ($n = 25$) (percent change of mean vs. control).

Extract	TEWL %		Erythema %	
	1 h	24 h	1 h	24 h
<i>Fucus vesiculosus</i>	-14.9	-11.3	-11.7	-20.7
<i>Undaria pinnatifida</i>	-14.0	-8.4	-9.8	-18.2
Placebo	0.4	-1.2	-0.6	-7.9

3.8. Clinical Test of *Fucus vesiculosus* Extract for Age Spots, Brightness and Wrinkles

Results from the 20 subjects in the double-blind, placebo-controlled, hemi-face study showed that *Fucus vesiculosus* extract at 0.3% w/v was an effective cosmetic ingredient for reducing the melanin index of age spots, increasing brightness and decreasing wrinkles (Figures 2–4). The clinical evaluation was performed by a dermatologist as described in Section 2.8, with clinical scores assigned from the following: no variation, slight improvement, moderate improvement, or remarkable improvement. The percentage of subjects that experienced an improvement, as evaluated by the dermatologist, is noted in Table 13. According to the clinical analysis, after 60 days of use, 50% of the volunteers showed an improvement in skin brightness, 65% showed a reduction in skin spot appearance and 45% showed an improvement in the appearance of wrinkles. Whilst the trial was terminated at 60 days, the rate of reduction in age spot index and increase in brightness indicates marked trends. Intergroup analysis was performed by way of the Wilcoxon signed rank test for non-parametric data (Figures 2 and 3).

Table 13. Dermatologist clinical analysis of *Fucus vesiculosus* extract (percentage of subjects showing an improvement).

Metric	Treatment	Placebo	Treatment	Placebo	Treatment	Placebo
Variation vs. T0	T15 days	T15 days	T30 days	T30 days	T60 days	T60 days
Wrinkle evaluation	10%	0	30%	0	45%	5%
Reduction of skin spot appearance	0	0	40%	0	65%	0
Skin brightness	10%	0	40%	0	50%	0

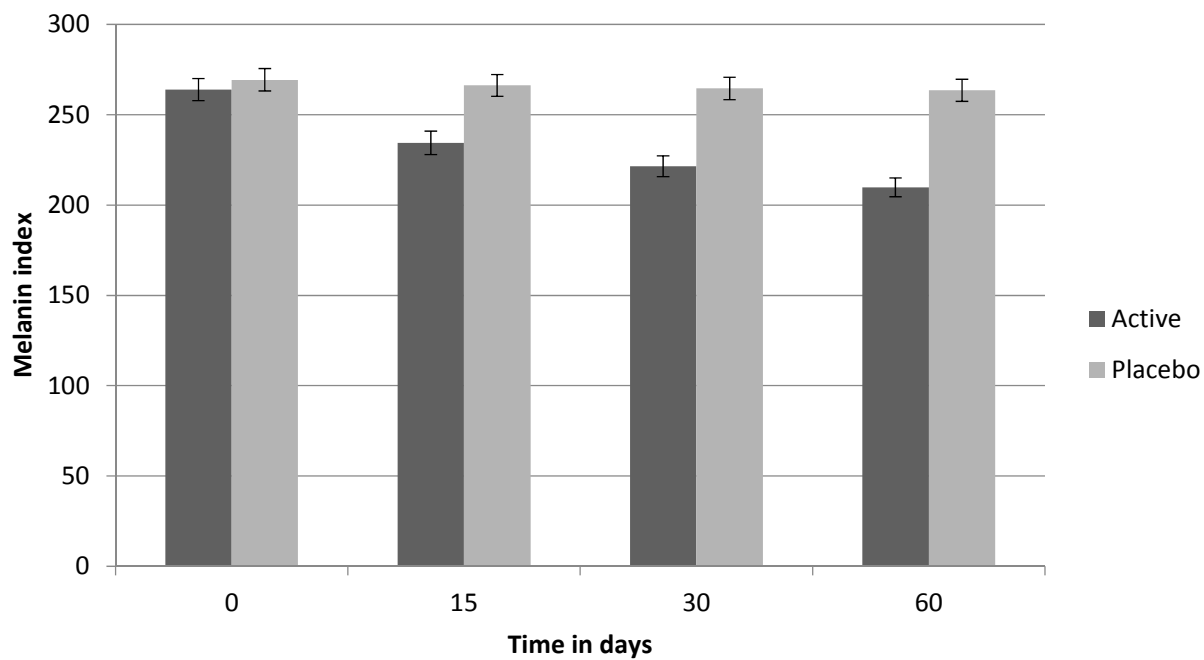


Figure 2. Clinical test on *Fucus vesiculosus* extract: age spot intensity over time.

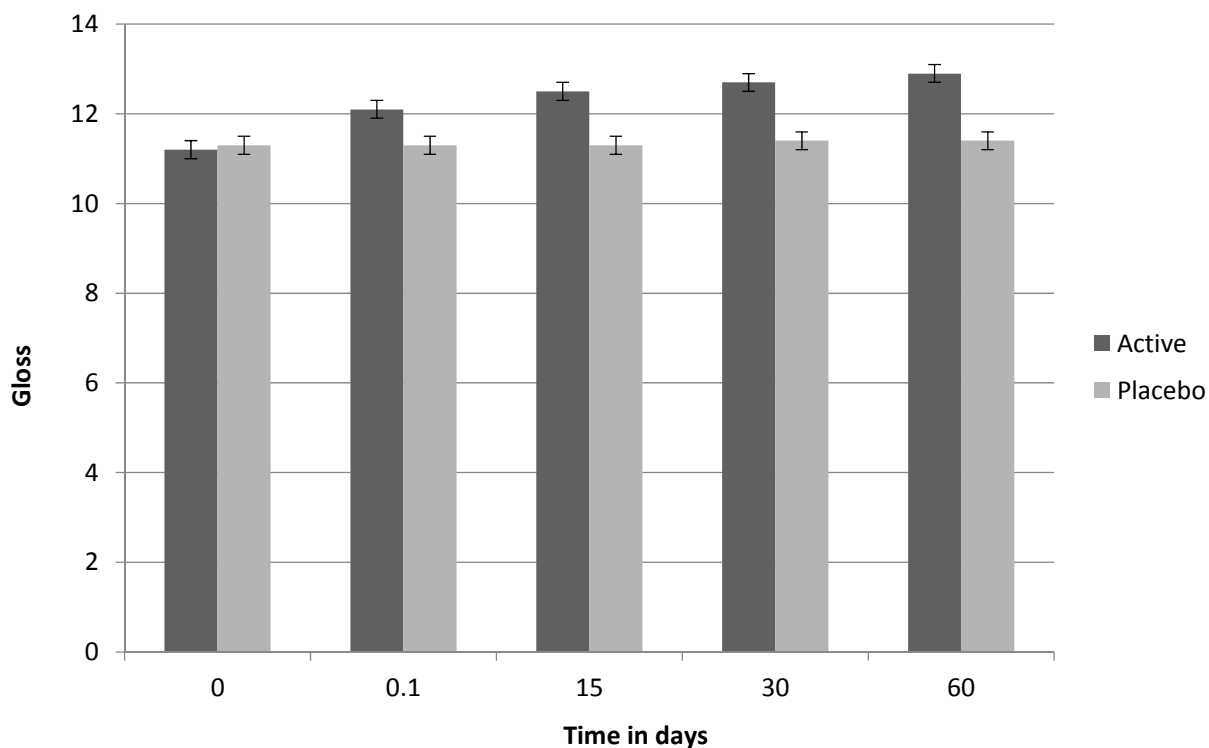


Figure 3. Clinical test on *Fucus vesiculosus* extract: brightness (gloss) factor, over time.

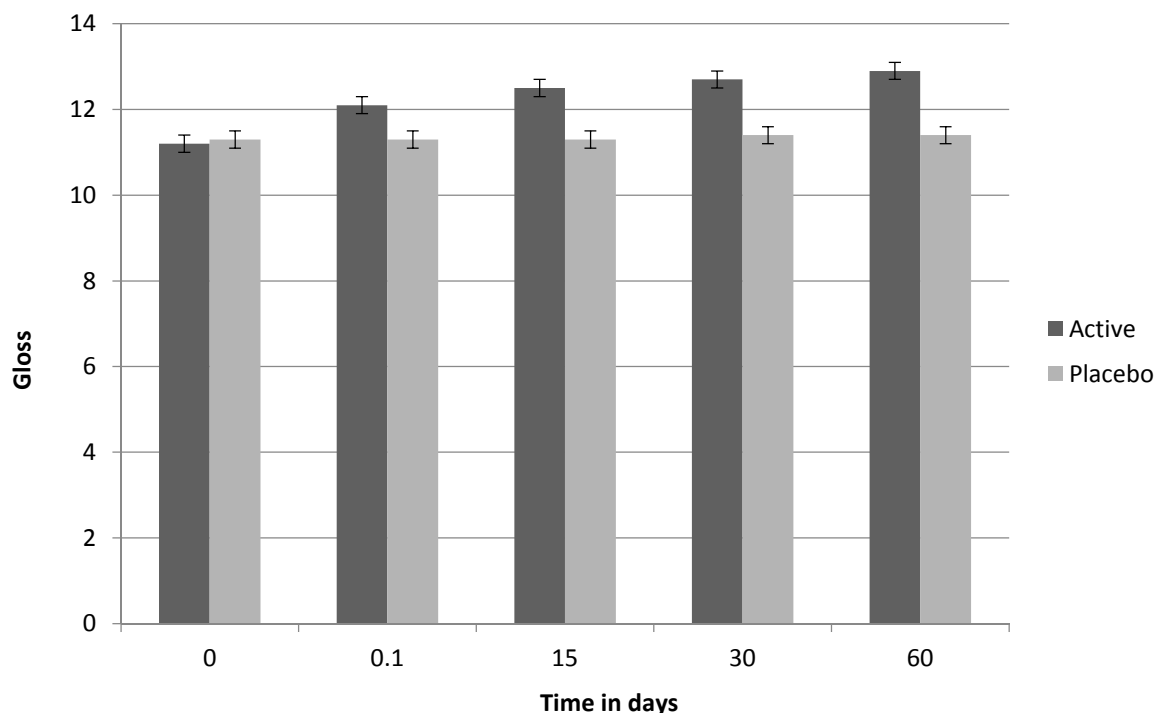


Figure 4. Clinical test on *Fucus vesiculosus* extract: wrinkle depth over time.

3.9. Discussion

Despite the popularity of marine algal extracts in cosmetic preparations, little has been reported to date regarding the specific bioactivity of these extracts. Over the past 20 years, there has been a growing body of *in vitro* research on the bioactive properties of fucoidans, and many of these studies have focused on the potential for fucoidan as a cosmetic ingredient. The key focus of research to date (including this study) has been to investigate the inhibitory effects of topically applied fucoidan on aging and photo-damaged skin. In this study, two extracts from two different marine algal sources were examined. The extracts were highly purified, extensively characterized fucoidan and polyphenol extracts, with purity accounting for upwards of 85% of the whole extracts. The purities were verified analytically, confirming the removal of many undesired components, such as salts and iodine.

The stimulation of skin matrix enzymes, including collagenase and elastase, is a key aspect of skin aging [11]. It is thought that the accumulation of degraded collagen fibrils prevents new tissue formation and causes further skin degradation by inducing further enzyme activity in a “positive feedback loop”. Lessening such enzyme activity may assist in the reduction of skin degradation and promote the formation of new matrix. Skin care applications for fucoidan as an immune regulator and as a soothing ingredient have been established in this study, with the *Undaria pinnatifida* extract. In this study, inhibition of collagenase (bacterial) and elastase (human neutrophil) by the *Undaria pinnatifida* extract *in vitro* was noted. Although it is not possible to directly infer effects from *in vitro* studies, early clinical observations demonstrated improvements in skin.

Further *in vitro* data for anti-aging applications for *Undaria pinnatifida* extract reported here includes *SIRT1* protein expression; changes in the gene expression profile of UV irradiated reconstructed human epidermis and inhibition of glycation, a marker for aging skin. Increasing the levels of *SIRT1* can mimic the benefits of caloric restriction, enhancing sugar and lipid metabolism,

and maintaining a younger physiology [19,20]. Sirtuins are expressed in human skin, and play complex roles in cellular metabolism [19]. In the skin, *SIRT1* levels are depressed by UV irradiation and oxidative damage and, in other studies, *SIRT1* stimulating extracts have also been effective in addressing skin aging [21]. Although it is not possible to directly extrapolate from *in vitro* studies, increasing the levels of *SIRT1* may assist in maintaining skin function, by reversing the effects of external factors. Whilst we demonstrated that *SIRT1* protein levels were increased *in vitro* by both extracts, the gene expression of *SIRT1* was not assessed in the reconstructed epidermis analysis described in this paper. This would clearly be a valuable marker in future studies of either *Undaria pinnatifida* extract or *Fucus vesiculosus* extract.

The role of fucoidan in UV protection may be explained by *in vitro* observations of the activation of Toll-like receptors (genes associated with the expression of antimicrobial peptides that are critical to innate immunity). Genes for Toll-like receptors 2 and 3 were strongly activated, at 387% and 229%, respectively. There was a large increase in the expression of wound-healing genes for the main matrix metalloproteases: at four hours, gene expression in skin treated with the *Undaria pinnatifida* extract was more than doubled as compared to control, showing an enhanced wound-healing signal. The stimulation of Toll-like receptors by other types of fucoidan has been demonstrated in cell culture [22]. The increased expression of extracellular matrix enzyme genes *in vitro* by the *Undaria pinnatifida* extract may be indicative of the ability of the extract to enhance the early wound-healing response, and then modulate that response. In addition, the decreased level of gene expression for *IL6* *in vitro* is noteworthy, with potential relevance to psoriasis [23]. The “quick response” defense activity may be cosmetically useful for enhancing dermal protection.

The polyphenol-rich *Fucus vesiculosus* extract was assessed in the ORAC5.0 assay, and demonstrated a marked total antioxidant value, with particular emphasis on inhibition of the superoxide free radical. Superoxide can be considered to be a precursor of the other free radicals, and this activity is useful in terms of topical cosmetic use as it can prevent environmental direct oxidative damage at the skin surface. Superoxide is not the strongest oxidant, as the hydroxyl radical is much more reactive [24]; however, superoxide is highly toxic to cells, and contributes to lipid and DNA damage. Antioxidants that scavenge superoxide ions help to prevent the formation of radicals such as hydrogen peroxide and the highly reactive hydroxyl species, thus preventing further tissue damage [11]. The polyphenol component of this extract may also be responsible for a higher expression of the *SIRT1* protein *in vitro*, in comparison to the *Undaria pinnatifida* extract.

In clinical applications, the *Fucus vesiculosus* extract exerted slightly superior soothing and protection results against UV damage when compared to the *Undaria pinnatifida* extract. *In vitro* measurements showed that the *Fucus vesiculosus* extract absorbed UV radiation in the skin-damaging UVA and UVB ranges, whereas the *Undaria pinnatifida* extract did not. *In vitro* testing demonstrated similar inhibition of the skin matrix enzyme human neutrophil elastase by both extracts; however, unlike the *Undaria pinnatifida* extract, *Fucus vesiculosus* extract was a highly effective inhibitor of mushroom tyrosinase as well as a highly effective antioxidant. As the tyrosinase examined in this study was mushroom-derived, it is not a directly comparable reflection of mammalian enzyme activity [25], but, nonetheless, provides indicative data.

Recently, a tyrosinase inhibitory fucoidan was isolated from kelp [26], with an apparent half-maximal inhibitory concentration of about 1 mg/mL. This is considerably higher than the

inhibitory concentrations observed here, which is likely attributable to the polyphenol content of the *Fucus vesiculosus* extract used in this study. Kang *et al.* isolated polyphloroglucinols from *Ecklonia stolonifera* [9] with half-maximal inhibitory values in the $\mu\text{g/mL}$ range, closer to the values observed in this study. Another research group noted fucoidan-reversible inhibition of tyrosinase, which was related to copper binding sites [27]. Clinical examination of the *Fucus vesiculosus* extract used here verified the skin brightening potential and age spot pigmentation reduction. It is not possible to directly infer that tyrosinase inhibition is taking place, as the other activities of the extract, such as antioxidative, *SIRT1* increasing, or other unknown effects may have resulted in the clinical observations. The small reduction in wrinkle depth of 6% compared to a 2% decrease for the placebo may also be a result of inhibition of oxidative damage. It is possible that absorbance of the fucoidan into the surface of the skin occurred and that this contributed to the small anti-wrinkle effect; however, absorbance measurement was not within the scope of this study.

4. Conclusions

The results show the topical benefits of two well-characterized fucoidan-rich macroalgal extracts derived from *Undaria pinnatifida* and *Fucus vesiculosus*. Both extracts demonstrated marked inhibitory *in vitro* effects on enzymes related to skin aging and the non-enzymatic glycation process. Clinical testing established the efficacy of these extracts in a range of applications including soothing, protection from UV rays, and wrinkle depth reduction. The *Fucus vesiculosus* extract with high polyphenol content demonstrated additional efficacy in antioxidant and skin brightening applications.

Acknowledgments

The authors are employed by Marinova Pty Ltd. with the exception of Giorgio Dell'Acqua who provided scientific direction. Funding and materials were provided by Marinova Pty Ltd. located at Cambridge, Tasmania, Australia.

Author Contributions

J. Helen Fitton, Giorgio Dell'Acqua and Vicki-Anne Gardiner contributed scientific direction. Samuel S. Karpinić performed characterization of the extracts and contributed to the manuscript preparation. Damien N. Stringer provided scientific direction and performed characterization of the extracts. Emma Davis provided scientific direction and contributed to manuscript preparation.

Supplementary Information

Supplementary Information can be accessed at <http://www.mdpi.com/2079-9284/2/2/0066/s1>.

Conflicts of Interest

The authors, with the exception of Giorgio Dell'Acqua, are employed by Marinova Pty Ltd.

References

1. Fitton, J.H. Therapies from Fucoidan; multifunctional marine polymers. *Marine Drugs* **2011**, *9*, 1731–1760.
2. Moon, H.J.; Lee, S.R.; Shim, S.N.; Jeong, S.H.; Stonik, V.A.; Rasskazov, V.A.; Zvyagintseva, T.; Lee, Y.H. Fucoidan inhibits UVB-induced MMP-1 expression in human skin fibroblasts. *Biol. Pharm. Bull.* **2008**, *31*, 284–289.
3. Moon, H.J.; Lee, S.H.; Ku, M.J.; Yu, B.C.; Jeon, M.J.; Jeong, S.H.; Stonik, V.A.; Zvyagintseva, T.N.; Ermakova, S.P.; Lee, Y.H. Fucoidan inhibits UVB-induced MMP-1 promoter expression and down regulation of type I procollagen synthesis in human skin fibroblasts. *Eur. J. Dermatol.* **2009**, *19*, 129–134.
4. Moon, H.J.; Park, K.S.; Ku, M.J.; Lee, M.S.; Jeong, S.H.; Imbs, T.I.; Zvyagintseva, T.N.; Ermakova, S.P.; Lee, Y.H. Effect of *Costaria costata* fucoidan on expression of matrix metalloproteinase-1 promoter, mRNA, and protein. *J. Nat. Prod.* **2009**, *72*, 1731–1734.
5. Senni, K.; Gueniche, F.; Foucault-Bertaud, A.; Igondjo-Tchen, S.; Fioretti, F.; Collicec-Jouault, S.; Durand, P.; Guezennec, J.; Godeau, G.; Letourneur, D. Fucoidan a sulfated polysaccharide from brown algae is a potent modulator of connective tissue proteolysis. *Arch. Biochem. Biophys.* **2006**, *445*, 56–64.
6. Thring, T.S.; Hili, P.; Naughton, D.P. Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complement. Altern. Med.* **2009**, *9*, doi:10.1186/1472-6882-9-27.
7. Yang, J.H. Topical application of fucoidan improves atopic dermatitis symptoms in NC/Nga mice. *Phytother. Res.* **2012**, *26*, 1898–1903.
8. Hwang, H.; Chen, T.; Nines, R.G.; Shin, H.C.; Stoner, G.D. Photochemoprevention of UVB-induced skin carcinogenesis in SKH-1 mice by brown algae polyphenols. *Int. J. Cancer* **2006**, *119*, 2742–2749.
9. Kang, H.S.; Kim, H.R.; Byun, D.S.; Son, B.W.; Nam, T.J.; Choi, J.S. Tyrosinase inhibitors isolated from the edible brown alga *Ecklonia stolonifera*. *Arch. Pharm. Res.* **2004**, *27*, 1226–1232.
10. Shibata, T.; Ishimaru, K.; Kawaguchi, S.; Yoshikawa, H.; Hama, Y. Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae. *J. Appl. Phycol.* **2008**, *20*, 705–711.
11. Kohl, E.; Steinbauer, J.; Landthaler, M.; Szeimies, R.M. Skin ageing. *J. Eur. Acad. Dermatol. Venereol.* **2011**, *25*, 873–884.
12. Lee, K.K.; Kim, J.H.; Cho, J.J.; Choi, J.D. Inhibitory effects of 150 plant extracts on elastase activity, and their anti-inflammatory effects. *Int. J. Cosmet. Sci.* **1999**, *21*, 71–82.
13. Fling, M.; Horowitz, N.H.; Heinemann, S.F. The isolation and properties of crystalline tyrosinase from *Neurospora*. *J. Biolog. Chem.* **1963**, *238*, 2045–2053.
14. Kiho, T.; Usui, S.; Hirano, K.; Aizawa, K.; Inakuma, T. Tomato paste fraction inhibiting the formation of advanced glycation end-products. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 200–205.
15. Choi, S.-Y.; Jung, S.-H.; Lee, H.-S.; Park, K.-W.; Yun, B.-S.; Lee, K.-W. Glycation inhibitory activity and the identification of an active compound in *Plantago asiatica* extract. *Phytother. Res.* **2008**, *22*, 323–329.

16. Mullen, W.; Nemzer, B.; Ou, B.; Stalmach, A.; Hunter, J.; Clifford, M.N.; Combet, E. The antioxidant and chlorogenic acid profiles of whole coffee fruits are influenced by the extraction procedures. *J. Agric. Food Chem.* **2011**, *59*, 3754–3762.
17. Cellular Anti-aging Effect Analysis. Brunswick Laboratories, Southborough, MA, USA, 2014. Available online: <http://www.brunswicklabs.com/anti-aging> (accessed on 13 April 2015).
18. Poumay, Y.; Dupont, F.; Marcoux, S.; Leclercq-Smekens, M.; Herin, M.; Coquette, A. A simple reconstructed human epidermis: Preparation of the culture model and utilization in *in vitro* studies. *Arch. Dermatol. Res.* **2004**, *296*, 203–211.
19. Serravallo, M.; Jagdeo, J.; Glick, S.A.; Siegel, D.M.; Brody, N.I. Sirtuins in dermatology: Applications for future research and therapeutics. *Arch. Dermatol. Res.* **2013**, *305*, 269–282.
20. Graff, J.; Kahn, M.; Samiei, A.; Gao, J.; Ota, K.T.; Rei, D.; Tsai, L.H. A dietary regimen of caloric restriction or pharmacological activation of *SIRT1* to delay the onset of neurodegeneration. *J. Neurosci.* **2013**, *33*, 8951–8960.
21. Moreau, M.; Neveu, M.; Stephan, S.; Noblesse, E.; Nizard, C.; Sadick, N.S.; Schnebert, S.; Bonté, F.; Dumas, M.; Andre, P.; *et al.* Enhancing cell longevity for cosmetic application: A complementary approach. *J. Drugs Dermatol.* **2007**, *6*, 14–19.
22. Makarenkova, I.D.; Logunov, D.Y.; Tukhvatulin, A.I.; Semenova, I.B.; Besednova, N.N.; Zvyagintseva, T.N. Interactions between sulfated polysaccharides from sea brown algae and Toll-like receptors on HEK293 eukaryotic cells *in vitro*. *Bull. Exp. Biol. Med.* **2012**, *154*, 241–244.
23. Dowlatshahi, E.A.; van der Voort, E.A.; Arends, L.R.; Nijsten, T. Markers of systemic inflammation in psoriasis: A systematic review and meta-analysis. *Br. J. Dermatol.* **2013**, *169*, 266–282.
24. Zhang, L.; Huang, D.; Kondo, M.; Fan, E.; Ji, H.; Kou, Y.; Ou, B. Novel high-throughput assay for antioxidant capacity against superoxide anion. *J. Agric. Food Chem.* **2009**, *57*, 2661–2667.
25. Jeon, S.-H.; Kim, K.-H.; Koh, J.-U.; Kong, K.-H. Inhibitory effects on L-Dopa oxidation of tyrosinase by skin-whitening agents. *Bull. Korean Chem. Soc.* **2005**, *26*, 1135–1137.
26. Yu, P.; Sun, H. Purification of a fucoidan from kelp polysaccharide and its inhibitory kinetics for tyrosinase. *Carbohydr. Polym.* **2014**, *99*, 278–283.
27. Wang, Z.J.; Si, Y.X.; Oh, S.; Yang, J.M.; Yin, S.J.; Park, Y.D.; Lee, J.; Qian, G.-Y. The effect of fucoidan on tyrosinase: Computational molecular dynamics integrating inhibition kinetics. *J. Biomol. Struct. Dyn.* **2012**, *30*, 460–473.