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Fish gelatin and its applications in selected pharmaceutical aspects as alternative source to pork gelatin

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Abstract

The demand for gelatin for food and pharmaceutical application is increasing because it is one of the most widely used food ingredients. Its applications in food manufacturing are very wide-ranging including enhancing the elasticity, firmness and consistency of food products. Most commercial gelatin is presently sourced from beef bone, hide and pigskin and bones. It was reported that about 41% of the gelatin produced worldwide is basically from pigskin. Fish gelatin represents one of the important types of gelatins from other sources rather than mammalian gelatins and it may thus be considered as an alternative to mammalian gelatin for use in various food and pharmaceutical applications. In the last decades, the rising interest in halal products issues for Muslim populations become one of the main reasons for exploring different types of collagen and gelatin from different animal sources. This review summarizes gelatin in four areas: (1) pig gelatin and halal issue, (3) selected methods used in fish gelatin extraction, (3) selected functional properties of fish gelatin and (4) selected pharmaceutical and medical application of fish gelatin.

Key words: Gelatin, meat, collagen, extraction, skin, pig, bovine, fish.

Introduction

Gelatin is one of the most popular biopolymers. It is tasteless and colourless solid substance derived from the fibrous protein collagen. It is widely used in food, pharmaceutical, cosmetic and photographic applications because of its unique functional and technological properties [1]. In cosmetic and health care products, gelatin is used as a gelling ingredient in face creams, body lotions, shampoos, hair sprays, sun screens and bath salts and bubbles [2]. It is also used in encapsulation of different drug products [3-5].

In fish processing industry, fish skin is considered as a major by-product that causing pollution could provide a valuable source of gelatin [6]. Nagai and Suzuki [7] reported that fish skin contains a large amount of collagen. The values in the fish skin of Japanese sea-bass, chub mackerel and bullhead shark were 51.4%, 49.8% and 50.1% (dry basis), respectively. Generally, gelatin from porcine or pig sources is preferred over other types such as bovine gelatin or gelatin from cattle sources in most non-Muslim countries because it is cheaper and because of the fear of BSE (bovine spongiform encephalopathy) or mad cow disease which can be contracted through cow based proteins. In the past, Muslim minorities simply avoided products that did not meet their standards [8]. Halal product markets nowadays are facing threats, gaining better insights in consumers behaviour which become of interest to several stakeholders [8]. Nowadays Muslims are making their presence felt socially and politically and are requesting halal certified food products [9, 10]. In food, pharmaceutical and cosmetic products, the use of halal gelatin such as fish gelatin is a concern of the Muslim community. It has been reported that gelatin from pig sources occupy greater than 50% of the world's gelatin market[11].

Pig Gelatin and Halal Issue

From physical point of view, it is a tasteless and colourless solid substance that is used as a gelling agent in food, pharmaceutical and cosmetic products. For instance, gelatins derived from either porcine or bovine sources have the same look, taste and produced the same result [11]. Therefore, it is impossible to differentiate the source of gelatin in products simply from their physical appearances.

The application of pig in food and pharmaceuticals become an issue for some religious adherents such as the Muslim and Jewish communities [12, 13]. Muslim halal required gelatin from sources other than pigs such as cows and/or fish and from animals slaughtered ritually. On the other hand, Muslims do not eat or use products that contain gelatin derived from porcine sources. For these reasons many studies developed to distinguish the species of origin of gelatin. Hidaka and Liu [12] used pH drop method to distinguish between gelatin from bovine and porcine sources in terms of induction time and formation of amorphous calcium phosphate (ACP) and hydroxyapatite (HAP). They found that gelatin from bovine bone increased the induction time with an increase of its concentration. At the concentration of 0.5 mg/ml gelatin, it took 1.75 times longer induction time than the control. In contrast, porcine skin gelatin increased the induction time only 1.53 times even at a concentration of 2.0 mg/ml. However, the effects of gelatins in HAP transformation were biphasic. Bovine bone gelatin increased it until 0.5 mg/ml, but after that it decreased. The peak concentration was 0.5 mg/ml and the peak value was 32% higher than the control. In contrast, porcine skin gelatin had a peak value at the concentration of 4.0 mg/ml, and the peak value was 45% higher than the control, but both gelatins did not affect

the rate of ACP formation.

Principal component analysis (PCA) was conducted for differentiation between bovine and porcine gelatins [14]. Fourteen bovine and five porcine gelatins were examined. The analysis procedure involved complete hydrolysis of samples by classic acid hydrolysis in order to release their amino acid residues. Separation and determination of amino acids was achieved by reversed-phase (RP) using HPLC following pre-column derivatisation. Orthophtaldialdehyde (OPA) and 4-chloro-7-nitro benzofurazane (NBD-Cl) were used as derivatisation reagents. Samples were compared using two-dimensional presentation graphs and it was found that the localization of the training set slightly changed and all the prediction samples fell into the right groups. The samples of bovine and porcine origin can be distinguished by a line in graphs, classification of stated components and some further parameters of the peak report table such as height, width and area percentage individually. The authors concluded that PCA on matrix of height, width and total matrix resulted in good differentiation between bovine and porcine gelatins.

Venien and Levieux [13] conducted an Enzyme-Linked ImmunoSorbent Assay (ELISA) which is a laboratory test used to detect antibody or antigen in the blood for differentiation of bovine from porcine gelatins using polyclonal anti-peptide antibodies. To obtain bovine-specific antibodies, the authors immunized rabbits against putative species-specific sequences of the bovine collagen alpha 1 (I) chain. Using these antibodies, an indirect ELISA was developed to allow a quick and easy differentiation between bovine and porcine gelatins. Moreover, a competitive indirect ELISA was done and it was found suitable to detect bovine gelatin and porcine gelatin using dilution of 2–4 parts per 1000 with Chorionic villus sampling (CVs) ranging from 5.7 to 7.7%. When testing mixtures of the largest possible range of mixtures of bovine and porcine gelatins (skin/hides or bones origin, acid or alkaline processes, high or low bloom) the detection limit should be down to a dilution of 8 parts per 100 bovine gelatin in porcine gelatin. The authors concluded that ELISA could be routinely used by pharmaceutical and food manufacturers to secure their supply chain.

Fourier transform infrared (FTIR) spectroscopy as a rapid method was used by Hashim *et al*. [15] to differentiate and authenticate source of gelatin whether from porcine or bovine. A simple and rapid method for qualitative determination was developed using FTIR in combination with attenuated total reflectance (ATR) and discriminate analysis. The spectra were analysed using a chemometric method, principal component analysis (PCA), to classify and characterise gelatin compounds using regions of the FTIR spectra in the range of 3290 – 3280 cm^{-1} and 1660–1200 cm^{-1} as calibration models. The results from PCA, which were subsequently represented by the Cooman's plot showed a clear distinction between gelatin samples of bovine and porcine origins. This qualitative approach can be used in addition of providing a rapid determination of the source of gelatin, may also be suggested as good tool to alleviate any doubt of the gelatin source for applications in both food and pharmaceutical industries.

Qian *et al*. [16] used high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) to identify the peptides originate from bovine, porcine and fish collagen type I in the digest mixtures. The sequence comparison indicated that bovine, porcine and fish collagen type I contain differential sequences, which might be used as marker peptides for gelatin differentiation. Bovine, porcine and fish gelatins were digested by trypsin. However, marker peptides specific for fish collagen type I were found in the digested fish gelatin. Actual sample analysis indicated that fish gelatin might be identified according to the marker peptides detected. The results demonstrated that detection of marker peptides using tandem mass spectrometry coupled with the digestion is a possible method for differentiation of fish gelatin from bovine or porcine gelatin.

Selected Methods Used in Fish Gelatin Extraction

Fish gelatin has the characteristics of high molecular weight, high viscosity, good transparency, very low heavy metal content. It has high water absorption and viscosity and is rich in amino acids. Table 1 summarizes the amino acid contents in fish gelatin as compared to those of pig gelatin. Several methods in many research works were conducted in order to extract gelatin from skin of different fish species for different purposes [17-31].

Generally and according to the method described by Songchotikunpan *et al*. [29], the extraction of gelatin from skin of fish can be achieved following this procedure: skin is washed thoroughly with running tap water for ~1 h to remove superfluous material. It is then soaked in 0.4 (w/v) NaOH aqueous solution for 4 h at room temperature (~25±1°C) at the skin/solution ratio of 1:7 (w/v). The skin is washed again with running tap water for ~1 h and later soaked in 0.4 (v/v) HCl aqueous solution for 4 h at room temperature at the same skin/solution ratio. The skin is then washed again with running tap water until pH is neutral. Finally, the skin is extracted with distilled water for 1.5 h at 70°C at the skin/water ratio of 1:2 (w/v). The extract was filtered through two layers of cheese clothes and evaporated at 70°C to remove ~70% of water. The filtrate was dried in a hot-air oven at ~50°C.

Gudmundsson and Hafsteinsson [36] used two fish species,

Table 1. Amino acid content in some fish gelatins compared to pork gelatin (residues/1000 total amino acid residues).

Amino acid	Cod skin[a]	Alaska pollock skin[b]	Hake[a]	Megrim[a]	Tilapia skin[c]	Pork skin
Alanine	96	108	119	123	123	112
Arginine	56	51	54	54	47	49
Aspartic acid	52	51	49	48	48	46
Cysteine	0	0	–	–	0	0
Glutamic acid	78	74	74	72	69	72
Glycine	344	358	331	350	347	330
Histidine	8	8	10	8	6	4
Hydroxylysine	6	6	5	5	8	6
Hydroxyproline	50	55	59	60	79	91
Isoleucine	11	11	9	8	8	10
Leucine	22	20	23	21	23	24
Lysine	29	26	28	27	25	27
Methionine	17	16	15	13	9	4
Phenylalanine	16	12	15	14	13	14
Proline	106	95	114	115	119	132
Serine	64	63	49	41	35	35
Threonine	25	25	22	20	24	18
Tryptophan	0	0	–	–	0	0
Tyrosine	3	3	4	3	2	3
Valine	18	18	19	18	15	26
Imino acid	156	150	173	175	198	223

[a] Go´mez-Guille´n *et al*. [32] ; [b] Zhou *et al*. [33]; [c] Sarabia *et al*. [34]; [d] Eastoe and Leach [35]; Source: Karim and Bhat [1]

namely, sin croaker (*Johnius dussumieri*) and shortfin scad (*Decapterus macrosoma*) for gelatin extraction. Thawed skin was thoroughly cleaned and rinsed with excess water to remove superfluous material and treated by soaking with 0.2% (w/v) sodium hydroxide solution for 40 min. Then it was soaked with 0.2% (w/v) sulphuric acid for 40 min. This was followed by soaking with 1.0% (w/v) citric acid. After each soaking treatment, the skins were washed under running tap water until they had a pH of about 7. Each soaking and washing treatment was repeated three times with a total time of 2 h for each treatment. The ratio of skin to washing liquid used was 1 kg skin (wet weight) to 7 litres of acid or alkali solution for each treatment. The skins were then subjected to a final wash with distilled water to remove any residual matter. The final extraction was carried out in distilled water at controlled temperature within the range of 40 – 50°C for 12 h. The ratio used was 1 kg (weight of wet skin) to 3 litres of distilled water. The clear extract obtained was filtered in a Büchner funnel with a Whatman filter paper (no. 4), followed by evaporation under vacuum and freeze–drying. The samples were stored at –20°C until used.

Gómez-Guillén and Montero [37] extracted gelatin from megrim (*Lepidorhombus boscii*) using acid extractions method with 0.05 M formic, acetic, propionic, lactic, malic, tartaric and citric acid. This extraction was carried out after a pretreatment with 0.2 N NaOH (1:6 w/v) at 5°C (30 min). The skins of the fish were cleaned, stirred for 16–18 h at 20°C with different solutions of 0.05, 0.1 and 0.5 M acids. On the other hand, Gómez-Guillén *et al*. [38] applied high pressure at 250 and 400 MPa, for 10 or 20 min. to extract gelatin from Dover sole (*Solea vulgaris*) fish. Mild acid (50 mM acetic acid) was used for swelling step for 3 h and subsequent overnight (16 - 18 h) gelatin extraction in distilled water at moderate temperature (45°C) was conducted.

The skin of black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*) were used for gelatin extraction [39]. The fish skin was rinsed with excess water followed by soaking in 0.2% (w/v) NaOH solution (40 min). This was followed by soaking in 0.2% H_2SO_4 acid and 1.0% citric acid. Rinsing with distilled water was carried out between soakings followed by final extraction in distilled water at 45°C (12 h).

Bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) were also used for gelatin extraction[40]. The skins of the fishes were soaked in 0.2 M NaOH (1:10, w/v) at 4°C with gentle stirring. The skins were further washed with tap water until neutral basic pH and soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 3 h at room temperature (25°C) with gentle stirring to swell the collagenous material in fish skin matrix. Acid-treated skins were washed and swollen fish skins were soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45°C (12 h) with a continuous stirring to extract the gelatin.

Channel catfish (*Ictalurus punctatus*) was also used in gelatin extraction [41]. Pre-treatment was conducted with alkaline solution followed by an acid solution (NaOH and acetic acid). The cleaned skin (30 g) was treated with NaOH (1:6 w/v) for variable time. Then, samples were drained using cheesecloth and rinsed with tap water (related two times). Afterwards the samples were treated with acetic acid (1:6 w/v) for variable times, followed by draining using cheesecloth and rinsed with tap water (1:6 w/v) (three times) (samples maintained at 4°C). After the above pretreatment, ion-free water was added to the flasks and samples were extracted in a water bath for variable times.

Rahman *et al*. [42] extracted gelatin form yellowfin tuna (*Thunnus albacares*). For sample preparation and pre-treatment, the frozen fish skin was thawed at room temperature for 1 h. Skin was washed in running water and dipped in 0.5 M NaCl (5 min at 5°C), followed by washing in tap water (three times) and treating with 0.1 N NaCl. The solution was further washed (three times) with distilled water and placed in 0.1 N acetic acid solution. The extraction was achieved with water by heating and stirring at 50°C on a hot plate for 18 h.

Moreover, Arnesen and Gildberg [43] used Atlantic salmon (*Salmo salar*) and Atlantic cod for gelatin extraction. The skins of the fish were first frozen at -20°C. Gelatin was prepared by acid extraction method. The skins were washed in cold water (about 8°C) and incubated (twice) in cold NaOH solutions (0.04 N) (30 min) followed by acid incubations (twice) (30 min) in H_2SO_4 (0.12 M) and then in citric acid solution (0.005 M). After the final washing in cold water, a two-step gelatin extraction (each step 2 h) was performed by gentle stirring, first in 1 litre water at 56°C and then in 1 litre at 65°C.

Selected Functional Properties of Fish Gelatin

The functional properties of fish gelatin have been reported in several research works [44-51].Gelatin is used as a foaming, emulsifying, and wetting agent in pharmaceutical and medical applications because earlier studies [52] have shown that gelatin is surface-active and capable of acting as an emulsifier in oil-in-water emulsions. Cole [53] reported that the hydrophobic areas on the peptide chain are responsible for giving gelatin emulsifying and foaming properties. However, in the studies of Dickinson and Lopez [46, 52], gelatin was characterized generally as a weaker emulsifier than other surface-active substances such as globular proteins and gum arabic. Earlier, Toledano and Magdassi [54] have reported that during homogenization, gelatin often produces relatively large droplet sizes which can be hydrophobically modified by the attachment of nonpolar side-groups or can be used in conjunction with anionic surfactants to improve its effectiveness as an emulsifier [55, 56].

In most applications, gelatin is chosen not only for its surface-active properties but also of its unique low melting point which in the range of 10–30°C results in the melting of gelatin gels in the mouth [57]. Fish gelatin is also widely used in the production of films and all fish gelatins had excellent film–forming properties [40, 58, 59]. Jongjareonrak *et al*.[40] observed that gelatin films from the skins of a warm-water fish species exhibited stress and elongation at break similar to that of bovine bone gelatin. However, fish gelatin film also exhibited lower water vapor permeability characteristic than pigskin gelatin[59]. It was observed that fish gelatin films prepared from cold-water and warm-water fish also resulted in different water vapor permeability measurements [58]. The water vapor permeability of cold-water fish gelatin films was significantly lower than that of warm-water fish which was attributed to increased hydrophobicity. This can be explained in terms of the amino acid composition in different animal species [58].

Selected Pharmaceutical and Medical Application of Fish Gelatin

Gelatin is widely used in various pharmaceutical and medical applications [60-62]. However, it was noticed that there were increasing concerns with the continued use of animal-derived products such

as gelatin [63]. Gelatin is cheap, biodegradable and demonstrates good biocompatibility and has therefore numerous uses in the medical field, for example as capsules, slow release matrices, sponges, scaffolds and "smart" hydrogels [64-69]. The industrial utilization of collagen or gelatin obtained from non-mammalian species is growing up in research in the last decade. It is used in classical food, photographic, cosmetic and pharmaceutical application which is based mainly on gel-forming properties of gelatin [70].

Fish gelatin, especially that extracted from warm-water fish, possesses similar characteristics to porcine gelatin and may thus be considered as an alternative to mammalian gelatin for use in pharmaceutical products [1]. Fish gelatin with low melting points could be used in the microencapsulation of vitamins and other pharmaceutical additives such as azoxanthine [1]. According to van den Bosch [71] and Bigi [66], the gel-strength of gelatin depends on the average chain length as well as the extent of intact collagen chains, whereas the viscosity mainly depends on average chain length. Elvin *et al.* [69] used cold-water fish gelatin for surgical purposes (as surgical tissue sealants), and when the gelatin was tested *in vivo* in sheep lung, it effectively sealed a wound in lung tissue from blood and air leakage. The elastic properties of gelatin, thermal stability, speed of curing and high tissue adhesive strength, offer considerable improvement over the surgical tissue sealants. Table 2 summarizes selected pharmaceutical applications of fish gelatin from different species.

Conclusions

Muslim halal required gelatin from sources other than pigs such as cows and fishes because Muslims do not eat or use products that contain gelatin derived from pig sources. Many analytical procedures were used in order to characterize and identify different types of gelatin such as porcine, bovine and fish gelatins and among them principal component analysis (PCA), Fourier transform infrared (FTIR) and high performance liquid chromatography (HPLC) analyses. Processing conditions such as solvent, time and temperature used are important to produce the optimum yield and good quality of fish gelatin. The functional properties, such as foaming, emulsifying, wetting agent and elastic properties, thermal stability, speed of curing and high tissue adhesive strength, make fish gelatin unique material for different pharmaceutical applications.

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Table 2. Selected Pharmaceutical Application of Fish Gelatin from Different Species.

Type of fish gelatin	Sample preparation and treatment	Pharmaceutical application	Reference
●Codfish gelatin	●Thirty clinically fish-allergic patients (15F/15M) were included in this study (age 9–50 years, mean 29.9). All had been diagnosed allergic to codfish. Age at onset of fish allergy varied from <1 year to 20 years old, with most becoming allergic in childhood. All kept a strict diet avoiding fish and hidden fish according to the dietician's advice, but they reported immediate symptoms after accidental intake of codfish.	●Evaluation of allergenicity of commercial, food-grade fish gelatin.	Hansen *et al.* [72]
●Pacific codfish skins	● Male ICR mice were randomly divided into the following six groups (10 mice in each group): group a, normal group; group b, model group; group c, at dose of 50 mg kg^{-1} d^{-1} bw PEP1 group; group d, 200 mg kg^{-1} d^{-1} bw PEP1 group; group e, at dose of 50 mg kg^{-1} d^{-1} bw PEP2 group; group f, 200 mg kg^{-1} d^{-1} bw PEP2 group. Animals in the normal group and model group were given normal saline at the same volume. All animals were maintained on a 12 h light/dark cycle and fed a standardrodent chow diet. All mice, except the normal group, were irradiated with the same UV source.	Investigation of changes of antioxidase activity in skin tissue and the arrangement of collagen fibres using ultraviolet radiation-induced skin photoaging.	Hou *et al.* [73]
●Cod bone gelatin	● A total of 84 3-month old female Sprague Dawley rats were housed under standard laboratory conditions (temperature 21 ± 2 °C, humidity 55 ± 10%, lighting cycle 12 h light/12 h dark). All animals had free access to tap water and were fed AIN-93M diet. After 14 days of equilibration, these animals were either randomized (OVX, $n = 72$) or sham-operated (SHAM, $n = 12$). Briefly, rats were intraperitoneally (I.P.) anesthetized with sodium pentobarbital at a dose of 30 mg/kg. A transverse incision was made inferior to the rib cage on the dorsolateral body wall. The uterine tubeswere excised and clamped and the ovaries excised. The skin was closed with wound clips. Rats subjected to the SHAM surgical procedure only had the ovaries exteriorized and replaced. One week later, OVX rats were divided into 6 groups of 12 each: a distilled water-treated group and groups treated with cod bone gelatin at 0.375, 0.75, 1.5, 3, 6 g/kg weight, respectively. The cod bone gelatin dose was mixed with distilled water as a vehicle and administered orally daily	Investigation of gelatin on bone metabolism and bone microarchitecture in ovariectomized rats.	Han *et al.* [74]
● Shark gelatin	● Four-week-old female Wister rats ($n = 60$) were given free access to a modified AIN-93 diet containing 20% of casein for 3 d of acclimatization. The rats were housed in collective cages at 20 ± 2°C on a 12-h light/12-h dark cycle, with free access to water. The animals were subsequently fed a modified AIN-93 diet containing 3% casein (low-protein diet). Administrated ovalbumin and collagen were dissolved in water at 40 mg/mL and heated daily at 100°C for 5 min. On the ninth day of the experiment, 40 rats were ovariectomized (ovx) and 20 rats were sham-operated (sham). One week later, rats were administered shark gelatin at an oral dose of 10, 20, or 40 mg/100 g of body weight per day for 2 wk. The control groups were given ovalbumin at a dosage of 20 mg/100 g of body weight per day. Ten rats from the ovx group and seven or six rats from the sham group were used. Body weight and food intake were recorded once on the second day of the experiment. Surgical procedures and killing of the animals were performed.	Investigation of bone mineral density through oral administration of shark gelatin to ovariectomized rats.	Nomura *et al.* [75]
● Cold-water fish gelatin	● A photochemical process was used to cast pieces of crosslinked gelatin. The $[RuII(bpy)_3]^{2+}$ was prepared as a 50 mMstock solution, and the SPS as a 1 M solution, both in water. Mixtures of gelatin (typically 100–175 mg/mL), 1 mM $[RuII(bpy)_3]^{2+}$ and 20 mM SPS in phosphate-buffered saline, pH 7.4 (PBS) were dispensed into Teflon orPerspex moulds, and irradiated for 30 s at room temperature with a quartz tungsten halogen lamp (300–1200 nm, 300 W × 2) from a distance of 150 mm. A xenon endoscope lamp (350–800 nm, 300 W, Olympus CLV-160) and an LED dental curing lamp (460 nm, 1200 mW/cm^2 at source, 3 M Epilar FreeLight 2) were also used in some experiments as described below. For thermal stability testing, discs (13 mm × 2 mm) of 150 mg/mL porcine gelatin were cast in Teflon moulds and incubated in PBS at the designated temperature. The thermally set discs contained 1 mM $[RuII(bpy)_3]^{2+}$. The photopolymerised discs contained 1 mM $[RuII(bpy)_3]^{2+}$ and 20 mM SPS, and were photopolymerised for 30 s. The discs were incubated in 10 mL of PBS at 20 °C or 90 °C for up to 4 h. Cross link Tissue sealants in sealing the wound from leakage of blood.		
● Squid gelatin	● MCF-7human breast carcinoma and U87 glioma cell lines were grown at 37 °C in a 5% CO_2, 95% air humidified atmosphere, in DMEM-Ham's F12 medium (1:1, v:v, Gibco). The medium was supplemented with 5% heat inactivated Fetal Calf Serum (FCS, Dutscher) to which penicillin (100 U/mL) and streptomycin (100 µg/mL) had been added (Pan biotech GmbH). The cells grown in flasks were trypsinized, centrifuged at 800 rpm for 5 min and dissolved in fresh culture medium at 10^4 cells/90 µL. Subsequently a 90 µL volume of suspension cells (10,000 cells) was added to each well of a 96-well microplate and incubated at 37 °C for 24 h. In order to perform cytotoxicity screening of the hydrolysates, hydrolysate stock solution at a concentration of 10 mg/mL was prepared in PBS 0.1 M (pH 7.4), and diluted 10-fold in cell culture medium containing the cells. The microplate was then incubated at 37 °C for 24, 48 and 72 h, changing the culture medium every 24 h and adding the hydrolysate at a final concentration of 1 mg/mL. At the end of every incubation period, 15 µL of 5 mg/mL tetrazolium salt (MTT) solution was added to each well, and the plate was incubated for 3 h. To stop succinate-tetrazolium reductase activity and solubilize formazan crystals, 200 µL of dimethyl sulfoxide (DMSO) was then added to each well and kept at 37 °C for 1 h. Absorbance was read on a plate reader (VERSAmax, Molecular Devices, Saint Gregoire, France) at 570 nm. The data were analyzed to calculate the percentage of viability inhibition induced by the presence of hydrolysate in cell culture medium determined by the equation: Viability inhibition (%) = 100 − (AH × 100 / AC) where AH and AC are the absorbencies measured for cell viability in culture medium containing only hydrolysate or PBS respectively. Following the same procedure, the effect of addition of hydrolysates at the same time as the cancer cells at the beginning of the experiment was also evaluated. For this evaluation, the initial conditions of the culture medium and the added hydrolysates were maintained for 72 h.	Development of functional foods with potential anticancer capacities.	Alemán *et al.* [76]