



Preparation and anatomical distribution study of ^{67}Ga -alginate nanoparticles for SPECT purposes in rainbow trout (*Oncorhynchus mykiss*)

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Abstract. Ergosan contains 1% alginate acid extracted from two brown sea weeds. Little is known about the target organs and anatomical distribution of Ergosan (alginate acid) in fish. Therefore, feasibility of developing alginate acid nanoparticles to detect target organ in rainbow trout is interesting. To make nanoparticles, Ergosan extract (alginate acid) was irradiated at 30 kGy in a cobalt-60 irradiator and characterized by transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). Results from TEM images showed that particle sizes of irradiated alginate acid ranged from 30 to 70 nm. The FTIR results indicated that gamma irradiation had no significant influence on the basic structure of alginate acid. Later, alginate acid nanoparticles were successively labelled with ^{67}Ga -gallium chloride. The biodistribution of irradiated Ergosan in normal rainbow trout showed highest uptake in intestine and kidney and then in liver and kidney at 4- and 24-h post injection, respectively. Single-photon emission computed tomography (SPECT) images also demonstrated target specific binding of the tracer at 4- and 24-h post injection. In conclusion, the feed supplemented with alginate acid nanoparticles enhanced SPECT images of gastrointestinal morphology and immunity system in normal rainbow trout.

Key words: rainbow trout • ^{67}Ga • intestine • SPECT • alginate acid nanoparticles • gamma irradiation

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Introduction

Ergosan contains 1% alginate acid extracted from two brown sea weeds, *Laminaria digitata* and *Ascophyllum nodosum*. Alginate acid is a high-molecular weight polymer of a repetitive unit containing D-mannosyluronic acid and L-gulosyluronic acid [1].

The small size of nanomaterials results in better surface functions of alginate acid nanoparticles as well as intestine cell permeability [2]. Different methods have been reported to synthesis of nanoparticles, such as interfacial polymerization, solvent evaporation, solvent deposition, nanoprecipitation, emulsification-diffusion and controlled gelification [3, 4]. Gamma irradiation of natural polysaccharides, such as chitosan, carrageenan and sodium alginate, offers a clean method for the formation of low-molecular weight oligomers. These oligomers have valid applications as antibiotic, antioxidant and plant-growth promoting substances [5, 6]. There are a lot of studies on the application of these degraded polysaccharides in different fields, such as agriculture [5, 7]. Moreover, many researchers had mainly focused on the effect of Ergosan (alginate acid) on fish growth, survival rate, reproductive performance, gastrointestinal morphology and innate immunity in serum and epidermal mucus [8, 9].

Table 1. Nuclear characteristics of three Ga isotopes which may be used as radiopharmaceutical labels

Characteristic	⁶⁷ Ga	⁶⁸ Ga	⁶⁶ Ga
γ Photon energy [keV] and abundance [%]	93(37%), 184(20.4%), 300(16.6%), 394(4.64%)	511(β+) (178%), 1077(3.0%)	511(β+) (114%), 834(6.03%), 1039(37.9%), 2752(23.2%)
Electron(s) energy [keV]	84, 92	1900(β+)	4153(β+)
Half-life	78 h	68 months	9.49 h
Decay mode	EC to ⁶⁷ Zn	10% EC+90% β+ to ⁶⁸ Zn	43% EC+57% β+ to ⁶⁶ Zn
Main production method	⁶⁸ Zn(p,2n) ⁶⁷ Ga	Daughter of ⁶⁸ Ge, ⁶⁶ Zn(α,2n) ⁶⁸ Ge	⁶⁶ Zn(p,n) ⁶⁶ Ga
Natural abundance of target	18%	28%	28%
Contaminant	⁶⁶ Ga, ⁶⁵ Zn	⁶⁸ Ge	⁶⁵ Zn
Beam energy [MeV]	22→12	40→20	15→6

Even though, little is known about the target organs and anatomical distribution of Ergosan (alginic acid) in fish. Therefore, feasibility of developing alginic acid nanoparticles to detect target organ in rainbow trout is interesting.

SPECT is a powerful and non-invasive imaging technique to visualize the biodistribution of molecules labelled with radioactive isotopes such as ⁶⁷Ga, ^{99m}Tc and ¹²⁵I [10]. The 78.3 h physical half-life and a rather good detectability of its photon emission make gallium-67 one of the most suitable nuclides for radiopharmaceutical research [11]. Table 1 shows characteristics of three Ga radioisotopes, which may be considered for this purpose.

Therefore, the objectives of this study were (1) characterizing gamma-irradiated Ergosan at 30 kGy as nanoparticles by transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR), (2) investigating intestinal uptake and anatomical distribution of ⁶⁷Ga alginic acid nanoparticles using single-photon emission computed tomography (SPECT) in rainbow trout.

Materials and methods

Fish

Healthy rainbow trout weighing 100–130 g raised from a fish farm in Karaj, Iran were transferred and kept in running water (flow rate 0.4 l/s) in polypropylene tanks (300 l) with water temperature $15 \pm 1^\circ\text{C}$, dissolved oxygen 5.2 ppm, and natural photoperiod (10 L:14 D).

Preparation of gamma-irradiated Ergosan

Commercial Ergosan (Schering-Plough Aquaculture™, UK) was suspended in sterile 0.15 M phosphate buffered saline (pH 7.2). Sample was sonicated for 30 min in a water bath sonicator (Jencons, England) and centrifuged at $5000 \times g$ for 15 min [12]. After precipitation in 2.5 volumes of 96% ethanol, Ergosan extract sample was dried at 40°C and then milled to the mesh size of 53–125 μm. Powdered Ergosan was irradiated at 30 kGy from cobalt-60 gamma irradiator (PX-30 Issledovatel, Russia) at a

dose rate of 0.22 Gy/s [13, 14]. Dosimetry was performed with Fricke reference standard dosimetry system after irradiation process; the irradiated-Ergosan extract was stored at 4°C for further experiments. The final irradiated-Ergosan extract powder prepared from 5 g crude Ergosan was 0.33 g.

Characterisation of gamma-irradiated Ergosan extracts (alginic acid)

Ergosan-irradiated particles were characterized by FTIR (KBr pellets on a Bruker spectrophotometer, EQUINOX 55, Germany) in the transmittance mode with a resolution of 4 cm^{-1} in a range of 400 to 4000 cm^{-1} .

The nanoparticles were stained with 2% phospho-tungstic acid solution and immobilised on formvar-coated grid. Particle sizes were confirmed using FEI/Philips EM 208S transmission electron microscope.

Production of ⁶⁷Ga

Production of ⁶⁷Ga was performed at the Nuclear Medical Research School (NARS), using enriched zinc-68 target in a 30 MeV cyclotron (Cyclone-30, IBA). The radiochemical procedure for ⁶⁷Ga is a two-step separation of ⁶⁷Ga from the enriched ⁶⁸Zn after dissolution of the irradiated target. This results in a 0.05 M HCl solution containing the non-carrier added ⁶⁷Ga as ⁶⁷GaCl₃. Final separation of ⁶⁷Ga from Zn is done by cation exchange chromatography using Dowex 50Wx8 (200–400 mesh, H⁺ form).

Radiolabelling of alginic acid nanoparticles with ⁶⁷Ga

The alginic acid nanoparticles were labelled by using an optimized protocol according to the literature (Orlando *et al.* [15]), with minor modifications. Typically, ⁶⁷Ga-chloride (37–110 MBq activity, 0.2 M HCl) was added to a conical vial and dried under a flow of nitrogen. Then, phosphate buffer (1 ml, 0.1 M, pH 8) and alginic acid nanoparticle suspension (100 μl, 0.3 g/100 ml) was added and mixed gently for 30 s, respectively. The solution was

stirred at room temperature for 30 min. Following incubation, the efficiency of radiolabelled alginic acid nanoparticles was checked, using paper chromatography and HPLC methods, for the purity of the radiolabelled samples.

Quality control of ⁶⁷Ga-alginic acid nanoparticles

Paper chromatography: 5 µl of the sample was spotted on a chromatography paper (Whatman No. 1. Whatman, Maidstone, UK), and developed in a solvent containing 1 mM DTPA in DDH₂O as the mobile phase.

High-performance liquid chromatography: HPLC was performed on the final preparation using acetonitrile solution (1 mM, pH 8.5) as eluent (flow rate: 1 ml/min pressure: 130 kgF/cm²) for 28 min in order to elute low molecular weight components.

SPECT imaging of ⁶⁷Ga-alginic acid nanoparticles in rainbow trout

Images were taken at 4 and 24 h after injection of ⁶⁷Ga-alginic acid nanoparticles by a dual-head gamma camera system (model: DST-XL made by SMV company). The collimator used was MEAP (medium energy all purpose) at the head toward gantry position with the planar-static acquisition type.

Anatomical distribution of ⁶⁷Ga-alginic acid nanoparticles in normal rainbow trout

To determine the anatomical distribution, ⁶⁷Ga-alginic acid nanoparticles were administered to the normal rainbow trout. A volume (100 µl) of the final ⁶⁷Ga-alginic acid nanoparticles solution containing 3.7 MBq ⁶⁷Ga was injected intra-peritoneally to the rainbow trout. The fishes were killed at exact time intervals (4 and 24 h), different organs (liver, kidney, heart, blood, spleen, intestine, skin, pyloric and gill) were taken, washed by normal saline, dried on filter paper and weighed. After weighing, specific activities were calculated as the percentage of the 184 keV peak area per gram of tissue. The 184 keV peak intensities were measured with a gamma-ray scintillation counter. For better comparison, the anatomical distribution of free ⁶⁷GaCl₃ was also determined. The radioactivity concentration was expressed as SUV (standardized uptake values):

$$\text{SUV} = \frac{\text{organ activity/organ weight}}{\text{total given radioactivity/rainbow trout body weight}}$$

In order to further assess the extent to which normal rainbow trout organs could uptake free ⁶⁷GaCl₂ and ⁶⁷Ga-alginic acid nanoparticles, we performed anatomical distribution analysis of free ⁶⁷GaCl₂ and ⁶⁷Ga-alginic acid nanoparticles in two groups of normal rainbow trout. 0.1 ml of ⁶⁷Ga-alginic acid nanoparticles solution (containing 100 µCi radioactivities) was injected intra-peritoneally.

The total amount of radioactivity injected into each rainbow trout was measured by counting a 1-ml syringe before and after injection in a dose calibrator (model: CRC-15R, made by Capintec company) with a fixed geometry. The rainbow trout were selected and sacrificed by clove oil at selected times and the radioactivity was quantified in tissues.

Results

The Fourier transform infrared (FTIR) spectra of the irradiated and non-irradiated Ergosan extract (alginic acid) were recorded and compared (Fig. 1a) for typical absorption bands of carbohydrate backbone. Stretching vibrations of alginate appeared in the range of 3000–3600 cm⁻¹ for O–H bonds and 2920–2850 cm⁻¹ for aliphatic C–H bonds in irradiated Ergosan extract.

The bands observed at 1649 and 1460 cm⁻¹ were attributed to asymmetric and symmetric stretching vibrations of carboxyl salt ion, respectively. The latter is quite intense and was used for the characterization of alginate structure from its derivatives and ingredients (Table 2). The absorption band at 3400 cm⁻¹ is due to O–H stretching vibration, which normally ranges from 3700 to 3500 cm⁻¹. The absorption band at 2944 cm⁻¹ for irradiated Ergosan extract (alginic acid) corresponds to C–H stretching. The –CH₂ symmetrical stretching band was found at 1327–1378 cm⁻¹. Broad absorption bands in the range of 1100–990 cm⁻¹ corresponded to C–O stretching frequencies of the C=O and C–O–H groups in the glycoside ring of samples. In addition, broad and weak bands occurred in the range of 930–600 cm⁻¹, probably arising from out-of-plane deformations of the ring C–H and ring-bonded O–H groups. The peaks at 1637 and 1425 cm⁻¹ indicate for substitution (ionization) of the carboxyl group in molecular chains. Consequently, formation of the –COO⁻ group would give rise to resonance effect between the two C–O bonds. Indeed the characteristic carbonyl absorption

Table 2. FTIR absorption regions and band assignments for irradiated (a) and non-irradiated Ergosan extract (alginic acid) (b)

Wave number [cm ⁻¹]		Assignment
a	b	
3458	3557	OH stretching
2909	2944	CH stretching of CH ₂ and CH ₃ groups
1628	1637	H–O–H bending of absorbed water and/or C=O stretching of amid/or COO ⁻ asymmetrical stretching carboxyl groups
1423	1425	CH ₂ bending or OH in plane bending/or COO ⁻ symmetrical stretching carboxyl groups
1033	1033	C–O symmetric stretching of primary alcohol

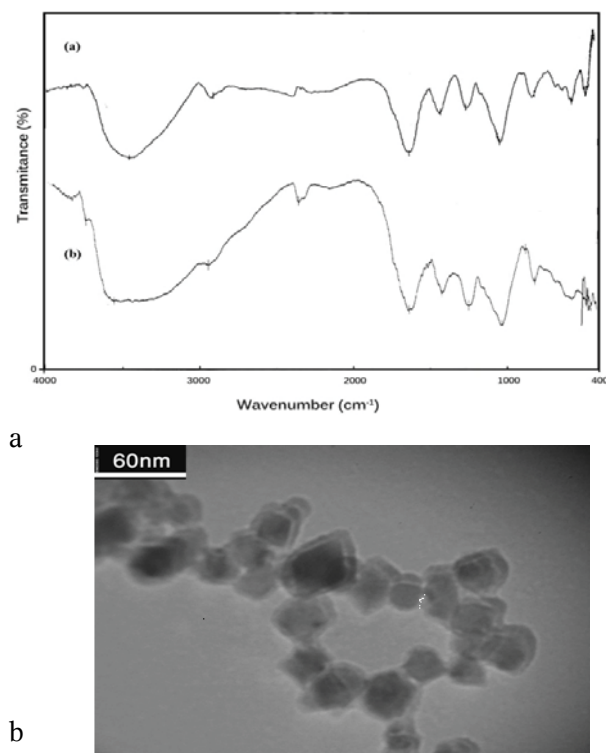


Fig. 1. (a) FTIR spectra of irradiated Ergosan extract (alginate) (a) and crude Ergosan extract (alginate) (b); (b) TEM image of Ergosan extract (alginate) after irradiation.

was replaced by two bands between 1637 and 1425 cm^{-1} and between 1400 and 1300 cm^{-1} , which corresponds to the asymmetrical and symmetrical vibrations of the $-\text{COO}^-$ structure. The characteristic absorption band of C–O stretching was found at 1033 cm^{-1} .

Micrograph of Ergosan extract (alginate) particles after gamma irradiation is presented in Fig. 1b. TEM analysis showed that the size of Ergosan extract (alginate) particles was within the 30–70 nm range. Nanoparticles were mostly spherical or oval in morphology.

The labelling of ^{67}Ga -alginate nanoparticles has been studied in order to investigate the uptake of ^{67}Ga -alginate nanoparticles by different organs in normal rainbow trout. The alginate nanoparticles were mixed with $^{67}\text{GaCl}_3$ solution, vortexed and kept at room temperature. Small fractions were taken from this mixture and tested by paper chromatography to find the best time scale for labelling. The radiolabelling of alginate nanoparticles reached to 97% after 60 min. Figure 2 demonstrates the paper chromatography scheme of free $^{67}\text{GaCl}_3$ and radiolabelled alginate nanoparticles.

At this stage, the mixture was tested by HPLC in order to determine the radiochemical purity before injection in rainbow trout. Figure 3 shows the HPLC chromatogram of ^{67}Ga alginate nanoparticles. The fast eluting component (2.55 min) was shown to be a mixture of free $^{67}\text{GaCl}_2$ and ^{67}Ga alginate nanoparticles, which were washed out on reversed phase–stationary phase. The radiolabelled carbohydrate was finally washed out in 28 min.

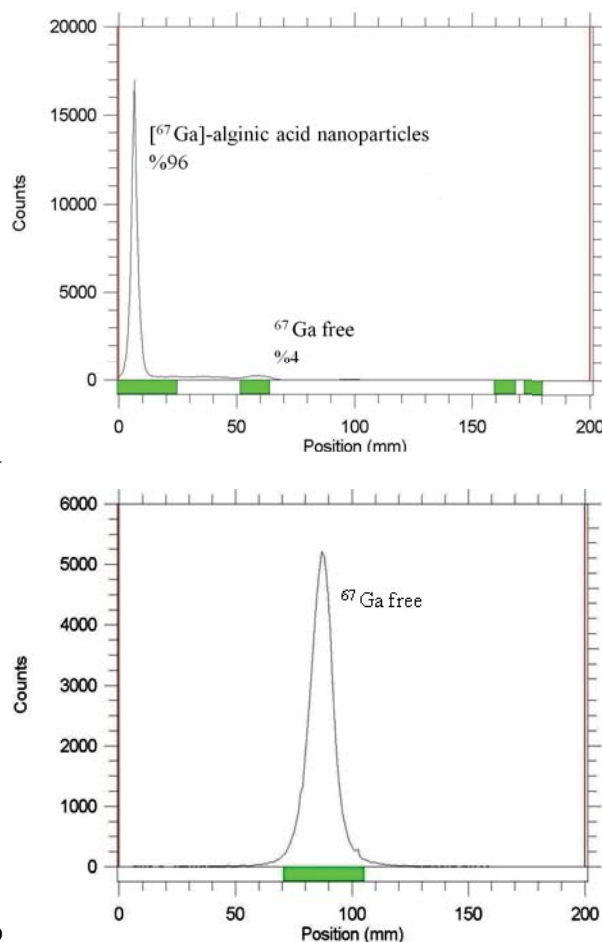


Fig. 2. RTLC of (a) ^{67}Ga -alginate nanoparticle and (b) ^{67}Ga free in 1 mM DTPA in DDH_2O as mobile phase.

Rainbow trout showed the uptake of free ^{67}Ga and ^{67}Ga -alginate nanoparticles in the skin, blood, heart, gill, intestine, pyloric, spleen, liver and kidney at 4 and 24 h following intra-peritoneal injection (Fig. 4).

The measurement of the organ radioactivity after intra-peritoneal administration of labelled alginate nanoparticles showed highest values in intestine (average SUV of all three time points is 9.64) followed by kidney, spleen and liver at 4 h post injection. After 24 h, the major tissues of interest were liver (average SUV of all three time points is 4.31)

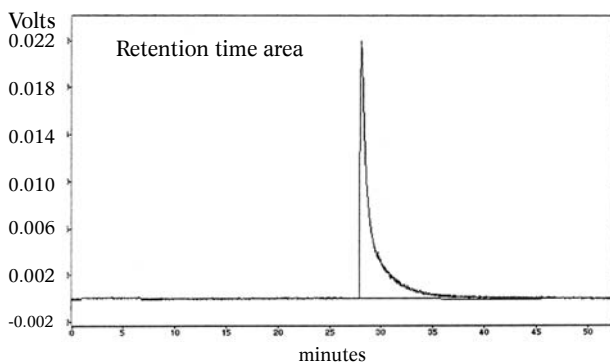


Fig. 3. HPLC chromatogram of final radiolabelled solution on a reversed phase column using a gradient of acetonitrile buffer.

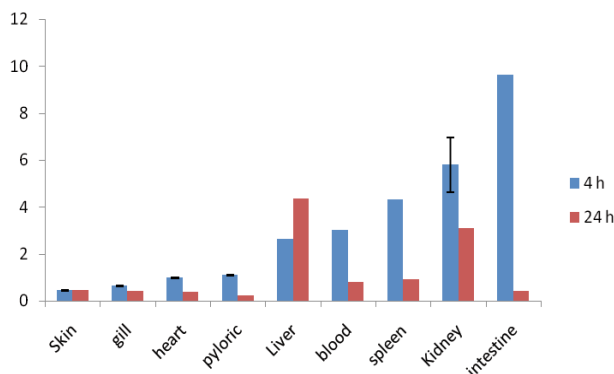


Fig. 4. Organ concentrations of radioactivity (presented as SUV, mean \pm SD) obtained at 4 and 24 h after the injections of ^{67}Ga -alginate acid nanoparticles.

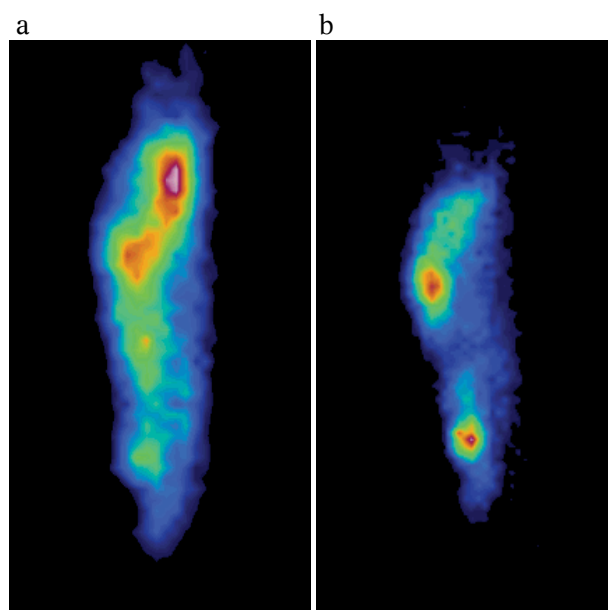


Fig. 5. SPECT images of ^{67}Ga -alginate acid nanoparticle in rainbow trout at 4 h (a) and 24 h (b) post injection.

and kidney, while the accumulated dose is negligible for other organs. The lowest uptake at 4 and 24 h post injection was observed in the skin, gill and heart with an SUV of 0.45, 0.63 and 0.98, respectively and in skin, pyloric and heart with SUV of 0.04, 0.2 and 0.3, respectively. SPECT images also demonstrated high GI accumulation of the tracer at 4 h and liver and kidney at 24 h (Fig. 5).

Discussion

Irradiated Ergosan extract (alginate acid) as a nanoparticles were labelled with $^{67}\text{GaCl}_2$, indicating the target organs and anatomical biodistribution of alginate acid in rainbow trout.

Recently, studies on polymeric nanoparticles were focused on their application in clinical diagnostics, therapeutics and carriers in delivery systems [16]. Gamma irradiation has been extensively used to generate nanoscale metals and nano-composites at room temperature and normal pressure [17]. In the present study, micrographs of alginate acid-irradiated granules obtained using TEM showed particle sizes

between 30–70 nm. Therefore, gamma irradiation at 30 kGy generated the nanoparticles from Ergosan extract (alginate acid).

To investigate whether any structural changes occurred during gamma irradiation, FTIR spectra were recorded. The bands concerning carboxyl groups (1649 and 1460 cm^{-1}) can be used to follow changes in the structure of different polymers of the alginate [18]. Negligible differences in the shape of this band before and after the irradiation were the result of -OH groups' participation in hydrogen bonds [19]. The shift of the band could be attributed to the weakening of hydrogen bonds [20]. The peaks, which indicate the presence of protein, were observed at approximately 1637 and 1628 cm^{-1} (amid I) in the irradiated and crude Ergosan extract (alginate acid), respectively. These spectra indicate that proteins present in Ergosan extract are covalently bonded to polysaccharides. However, stronger amid I band observed in the spectrum of irradiated Ergosan extract might be attributed to higher protein content in this substance. In general, results showed that both the irradiated and crude Ergosan extract had a similar pattern of FTIR spectra, typical of polysaccharides, without any notable changes in the functional group status.

This study is the first report on ^{67}Ga -labelled Ergosan extract (alginate acid) nanoparticles being used for preliminary anatomical distribution studies, based on previous experiences on the preparation of radiolabelled (^{125}I) alginate acid [15]. So far, little was known about the mechanism of the Ergosan (alginate acid) uptake in rainbow trout. The SPECT detected higher net uptake of ^{67}Ga -alginate acid nanoparticles in the intestine and kidney after 4 h and in liver and kidney after 24 h post injection, compared to the other organs in normal rainbow trout.

Previous investigations on the application of Ergosan (alginate acid) showed that this agent improved growth parameters and feed intake in rainbow trout [9] and Beluga sturgeon (*Huso huso*) [8, 21]. Also, Ergosan increased the density of the intestinal goblet cells, villus and fold length in rainbow trout [9] and villus, fold and enterocyte height in tilapia [22]. The results of the current study prove the positive effects of Ergosan (alginate acid) on imaging of the fish gastrointestinal morphology.

Moreover, the morphology of the immune system is different between fish and mammals. Instead of bone marrow and lymph nodes, the head kidney serves as a major lymphoid organ, in addition to the thymus and spleen in fish [23]. Accordingly, previous studies conducted on the use of Ergosan (alginate acid) in vaccine formulations had given very good antibody responses due to stimulation of lymphocyte proliferation [21, 24–26] and increased liver cytokine gene expression [27]. In addition, alginate acid and fucoidan can stimulate some cellular immune responses of head kidney leukocytes in cod, *Gadus morhua* [28]. Such studies are in agreement with the present study results, that ^{67}Ga -labelled alginate acid nanoparticles have increased uptake in kidney, spleen and liver at 4 h and liver and kidney at 24 h post injection, respectively.

Thus, above investigation demonstrates the potential of ^{67}Ga -labelled Ergosan extract (alginate

acid) nanoparticles for scintigraphic imaging of immuno receptor-expression, biodistribution and immunostimulants in rainbow trout. Furthermore, the supplemented feed with Ergosan extract (alginic acid) nanoparticles seems to enhance gastrointestinal and immune system SPECT images in normal rainbow trout.

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