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# The Preparation, Anti-inflammatory, and Antioxidant Properties of Glucosamine Hydrochloride from the Waste of *Litopenaeus vannamei* Processing Plant

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**ABSTRACT**

**Aims:** The purpose of the present study was to investigate the antioxidant and anti-inflammatory properties of glucosamine hydrochloride (G-HCl), glucosamine sulfate sodium chloride (GS-Na), and glucosamine sulfate potassium chloride (GS-K) isolated from the shells of *Litopenaeus vannamei* obtained from a shrimp processing plant.

**Materials & Methods:** G-HCl was synthesized via hydrolysis of chitin with concentrated HCl, followed by several sequential decolorization, crystallization, and washing steps. Using G-HCl as the precursor, adding sodium and potassium sulfates at 40 ºC for one hour produced GS-Na and GS-K.

**Findings:** The yield of chitin was found to be 19.9%, and those of glucosamine products ranged between 75.5%-82.5%. The HPAEC-PAD indicated the presence of glucosamine monomers, as compared with commercial standard, with different elution times to that of glucose. The appearance of O-H, N-H, and C-O-C characteristic signals in the FT-IR spectra further supported successful glucosamine isolation. SEM images and EDX glucosamine spectra confirmed the samples' elemental compositions and polyhedral crystalline structures. DSC and TGA thermograms indicated endothermic and exothermic peaks specific to glucosamine products. Relatively low DPPH and ABTS radical scavenging activities and ferric-reducing power were obtained for all glucosamine products. All the glucosamine derivatives indicated an anti-inflammatory effect on LPS-simulated RAW264.7 cells.

**Conclusion:** Glucosamine products showed no cytotoxicity and down-regulated NO release in RAW264.7 murine macrophage cells induced by LPS. Overall, the present results indicated the successful production of glucosamine with antioxidant and anti-inflammatory properties from the waste of the L. vannamei processing plant.

**Keywords:** *Litopenaeus vannamei*; Shell; Chitin; Glucosamine; Antioxidant; Anti-inflammatory.

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## Introduction

Some aquaculture waste products or by-products contain many biomolecules, including polysaccharides, peptides, proteins, enzymes, vitamins, and minerals with a wide variety of antioxidant, anti-aging, anti-inflammatory, antimicrobial, and antitumor activity [1,2,3]. In recent years, crustacean aquaculture has become the world's largest seafood production sector . Shrimp is a crucial aquacultural product processed for meat and waste products [5]. The consumption demand for shrimp and shrimp products is increasing yearly [6]. Global shrimp production will increase to 7,28 million tons by 2025 [7]. *Litopenaeus vannamei* is cultured commercially as one of the important species in Iran. The average production of *Litopenaeus vannamei* was estimated at 57000 tons in 2021[8]. Shrimp processing side streams, including shells, heads, and viscera, have attracted mounting attention because of their indispensable by-products, including chitin, chitosan, glucosamine, and astaxanthin, with diverse applications in food, cosmetic, and pharmaceutical industries [9]. This ever-increasing interest in valorizing shrimp wastes to produce feed and food products could benefit environmental sustainability and a circular economy. Depending on the source species, chitin, protein, calcium phosphate, and calcium carbonate are the most critical components of shrimp shells [10,11,12]. Chitin, β-(1–4)-poly-N-acetyl-D-glucosamine, has received significant interest as a natural amino polysaccharide chiefly due to its non-toxic, biocompatibility and biodegradability nature [12,13]. It is one of the most abundant polysaccharides in nature, widely used as a raw material to produce chitosan and glucosamine [12]. Glucosamine, 2-amino-2-deoxy-D-glucose ubiquitously found in the matrix of shells of crustaceans and could be produced via various approaches, including chemical (e.g., acid hydrolysis), microbial fermentation, enzymatic and combined methodologies [14]. Different forms of glucosamines, such as glucosamine sulfate, glucosamine hydrochloride, and N-acetyl glucosamine, are commercially used as dietary supplements in treating osteoarthritis and knee and back pains [15,16,17]. Nutritional glucosamine supplements have been extensively used to treat osteoarthritis [17]. The high level of nitric oxide (NO) and phospholipase A2 as inflammatory molecules have been observed in the cartilage of osteoarthritis patients [19]. Glucosamine is believed to be suppressing pro-inflammatory mediators such as NO, IL-6, and TNF-α in chondrocytes [20,21,22]. Besides the above advantages, plenty of studies have exhibited the cellular antioxidant effect of glucosamine as a potential therapeutic for OA [23,24,25]. Glucosamine exhibited prominent protective properties on the chondrocytes and prevention of oxidative stress [26]. Oxidative stress occurs through increasing production of NO and reactive oxygen species (ROS) derived from superoxide [26,27]. Yan et al. [28] have reported glucosamine's antioxidant capacity and immunostimulatory effects in both in vitro and animal models. Few studies observed that glucosamine hydrochloride effectively inhibited reducing power and superoxide/hydroxyl radicals scavenging activity dose-dependently [29,30]. Thus, to utilize the wastes of shrimp processing plants and reduce their environmental impact, this study aimed to isolate the commercial grade of glucosamine using acid hydrolysis, then evaluate the antioxidant activity and immunostimulating properties of glucosamine hydrochloride and glucosamine sulfate to produce high added-value products.

## Material & Methods

### Materials

Pacific white shrimp (*Litopenaeus vannamei*) wastes were purchased from Shil Abzi



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Company, Golestan, Iran, and transferred to the laboratory in a sealed ice box. Samples were immersed in 1% sodium hydroxide for two hours at room temperature as pretreatment to remove flesh from shrimp shells. After completing the pretreatment process, shells were washed with tap water several times at the same temperature to separate sodium hydroxide liquid and eliminate the impurities. Cleaned shells were dried at 60 ºC in an oven and milled using a grinder. Afterward, the powder was sieved using a Sieve Shaker (74-µm mesh size). Finally, dried shrimp shell powder was packed in plastic bags and stored at a temperature approaching -20 ºC before extraction of glucosamine hydrochloride.

**Extraction of chitin**

Firstly, the dry powder (1 g) was chemically demineralized. The powder was mixed with 1M HCl by stirring magnetically for two h and then subjected to centrifugation (6080 g for 5 min at four ºC) to sediment demineralized shells. The supernatant was removed, and the residue was washed with distilled water for one hour under constant stirring. At the end of the treatment, the demineralization liquid was centrifuged at 6080 g for 5 min at four ºC. Secondly, the demineralized residue was mixed with 4 % NaOH under continuous stirring at 70 ºC for six hours to eliminate protein. The solution was centrifugated (6080 g for 5 min at 4 ºC) and washed by agitation using distilled water for 12 h. The centrifugation was performed in the same manner as previously described. The white resulting residue was dried at 60 ºC for three hours.

**Preparation of glucosamine hydrochloride**

Glucosamine hydrochloride was synthesized via hydrolysis of chitin with concentrated HCl by stirring under reflux conditions at 95 ºC for 75 min. The liquid-to-solid ratio was 10:1. The solution was kept in a freezer at -80 ºC for 24 h. The frozen sample was thawed at room temperature for one hour. After centrifugation at 9000 rpm for 5 minutes at four ºC, the wet powder was washed with 5 mL of 95% ethanol for 20 min and centrifuged as described. The white precipitate was dried at 50 ºC for 3 hours, and the resulting glucosamine hydrochloride was passed through a 30 mesh sieve (535 µm).

**Preparation of glucosamine sulfate sodium chloride and glucosamine sulfate potassium chloride**

G-HCl (3 g) was slowly dissolved in water (8 mL). After 5 min, 0.98 g of sodium sulfate or 1.21 g of potassium sulfate was added to the solution of G-HCl. The solution was stirred at 40 ºC for 1 hour. The liquid was kept in a freezer at -80 ºC for 24 hours. Samples were centrifuged for 5 min at 6080 g and four ºC after thawing. Precipitates were washed with 10 mL of 95% ethanol for 20 minutes to decolorize glucosamine sulfate. The white solid crystals were centrifuged under the same conditions described and dried at 50 ºC for three hours to produce GS-Na and GS-K.

**HPAEC–PAD analysis**

G-HCl, GS-Na, and GS-K were determined using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis. The HPAEC-PAD system consisted of a GS50 gradient pump, an AS50 chromatography organizer, an ED50 electrochemical detector, and a 4 × 250 $mm^2$ CarboPac PA1 analytical column (Dionex Corp., Sunnyvale, CA). Samples (20 mg) were dissolved in deionized water (5 mL) and filtered through a 0.22 µm syringe filter.

**Fourier transform infrared spectroscopy (FT-IR) analysis**

The functional groups of G-HCl, GS-Na, and G-S-K were determined via Fourier transform infrared spectroscopy using a Tensor 27 spectrometer (Bruker Instruments, Billerica, USA). Samples were prepared in



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0.5-1 mm of KBr layer. The spectra were carried out in the region from 500 to 4000 $cm^{-1}$ at a high spectral resolution (2 $cm^{-1}$) in the absorbance mode.

**SEM-EDX analysis**

G-HCl, GS-Na, and GS-K morphology were examined by scanning electron microscopy (SEM) (JSM-IT500HR, JEOL Co., Japan) operating at an accelerating voltage of 20 kV. Before imaging, a thin layer of gold was coated on the samples. Energy-dispersive X-ray analysis (EDX) (JSM-IT500HR, JEOL Co., Japan) was performed along with SEM to analyze the elemental contents of G-HCl and G.S.

**Thermal analysis of glucosamine**

Thermal properties of G-HCl, GS-Na, and GS-K were studied by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). TGA experiment was performed with 10 mg of samples. Samples were heated at 10 ºC.$min^{-1}$ from 30 to 900 ºC using TGA 500 (T.A. Instruments, New Castle, DE, USA) under a constant nitrogen flow of 25 mL.$min^{-1}$. The DSC analysis was conducted on a TA DSC 25 (T.A. Instruments, New Castle, USA) enriched nitrogen gas flow. 10 mg of samples were heated from 20 ºC to 400 ºC at ten ºC.$min^{-1}$.

**Cytotoxicity and anti-inflammatory of glucosamine**

The RAW 264.7 cells were cultivated onto a T25 flask containing an RPMI-1640 medium culture plus 10% (v/v) FBS. The cells (100 µL volume of $1 \times 10^4$ cells.$well^{-1}$) were transferred into a 96-well microplate. Various concentrations (50, 100, and 150 µg.$mL^{-1}$) of samples and LPS (1 µg.$mL^{-1}$) were chosen to mix with cell suspensions. Afterward, 100 µL of samples were added to the cells, maintained at 37 ºC for 18 h. After incubation, the culture medium was collected and mixed with Griess reagent [31]. The murine macrophage-derived nitric oxide (NO) was determined by comparing with an obtained standard curve of $NaNO_2$ (1-200 µmol). The absorbance of the solution was read at 450 nm. The absorbance values (A) were translated into a macrophage proliferation ratio, Eq. (1). (%) = At/Ac × 100, where At and Ac are the absorbance values of the samples and control, respectively.

**DPPH radical scavenging activity**

The DPPH radical scavenging activity of G-HCl, GS-NA, GS-K, and G-S was evaluated according to the slightly modified procedure of Brand Williams et al. [32]. 0.1 mM DPPH was dissolved in 100 µL of 95 % ethanol. 100 µL of samples and ascorbic acid as a standard (25 to 299 µg.$mL^{-1}$) were added to 100 µL of the ethanolic solution of DPPH into a 96-well microplate and incubated by wrapping the microplate in the dark for 30 minutes. The optical density was recorded with a microplate reader at 517 nm. DPPH radical scavenging activity was obtained using the following equation:

DPPH scavenging effect (%) $=[(A_c-A_s)/A_c] \times 100$ Eq. (2)

Where $A_s$ was the absorbance of samples and $A_c$ was the absorbance of the control.

**ABTS radical scavenging activity**

ABTS radical scavenging activity was determined based on the protocol previously described by Re et al. [33,34]. ABTS solution was prepared by dissolving ABTS and potassium persulfate in water to 7 mM and 2.45 mM concentrations, respectively. The solution was maintained in the dark for 16 hours and diluted with ethanol to obtain an optical density of 0.7 at 734 nm. Afterward, 150 µL of ABTS was added to 50 µL of each sample and ascorbic acid at various concentrations (25 to 200 µg.$mL^{-1}$) in a 96-well microplate. Samples were held in the dark for 20 min, and optical density was recorded at 734 nm using a microplate reader. The inhibition of ABTS radical scavenging was obtained according to the following equation:

ABTS scavenging activity (%) $= [(A_c-A_s)/A_c] \times 100$ Eq. (3)



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where $A_s$ and $A_c$ are the optical densities of the samples and control (150 µL of ABTS with 50 µL of ethanol), respectively.

**Reducing power activity**

The reducing power of G-HCl, GS-Na, GS-K, and G-S were determined by Oyaziu's method [35]. Initially, 500 µL of samples at concentrations of 25 to 200 µg.mL$^{-1}$ were transferred in a vial containing 500 µL of 1% potassium ferricyanide and 500 µL of 0.2 M sodium phosphate buffer (pH 6.6). The final mixture was incubated at 50 ºC for 20 min. Then, 500 µL TCA (10%) was added, and solutions were centrifuged at 11000 rpm for 10 min. Finally, 1 mL of supernatant was transferred to a new vial containing 1 mL distilled water and 200 µL of ferric chloride (0.1%) and allowed to stand for 10 min. The absorbance of the sample and standard were measured at 700 nm.

**Statistical analysis**

The experiment's results were analyzed using SPSS (version 17.0). The one-way analysis of variance and Duncan multiple range test were carried out at a significant level of 5%.

## Findings

**Production of chitin and glucosamine**

Chitin was isolated by a chemical procedure using hydrochloric acid and sodium hydroxide at different concentrations and temperatures to eliminate the minerals and proteins, respectively. As presented in Table 1, the weight of the dried chitin obtained in the present study was 19.90 ± 1.02%. G-HCl was prepared by acidic hydrolysis of chitin using hydrochloric acid. The overall yield of G-HCl, GS-Na, and GS-K were 82.52%, 75.53%, and 82.11%, respectively.

**HPAEC–PAD analyses**

The HPAEC-PAD method is one of the most efficient techniques for separating monosaccharides [36]. HPAEC-PAD analysis was performed to determine G-HCl, GS-Na, GS-K, G-S, glucose, and water. The HPAEC-PAD elution profile of components is shown in Figure 1. The CarboPac PA1 column was explicitly developed for this analysis. The results indicated that glucosamine-standard was separated in elution time of 5 minutes on a HPAEC column. G-HCl, GS-Na, and GS-K were liberated and appeared simultaneously (5 min). These results were further confirmed by the elution profile of glucose, which was observed in an elution time of 6 min. Glucosamine hydrochloride and glucosamine sulfate were identified by comparing them with G-S and glucose retention time.

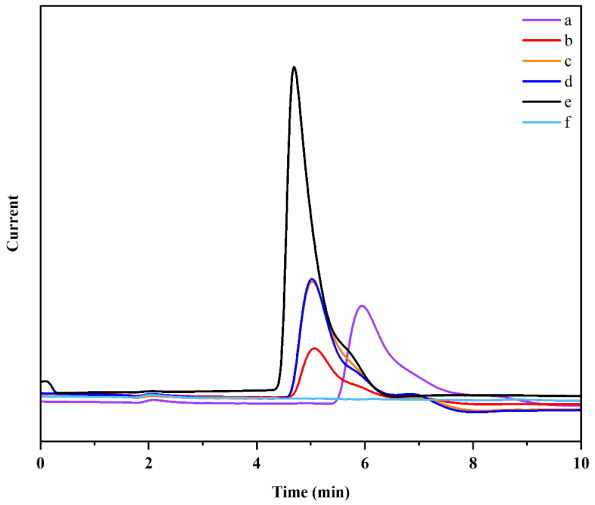


**Figure 1)** HPAEC-PAD of (a) glucose, (b) glucosamine hydrochloride, (c) glucosamine sulfate sodium chloride, (d) glucosamine sulfate potassium chloride, (e) glucosamine-standard and (f) water

**FT-IR spectroscopy**

FT-IR spectroscopy was conducted to determine the specific chemical bonds of G-HCl, GS-Na, and GS-K (Figure 2). Glucosamine exhibited peaks at around 2500-3500 cm$^{-1}$, which attributed to the N-H and O-H groups [37]. Zaeni et al. [38] indicated that the peaks in the spectrum at 3353 cm$^{-1}$ and 3293 cm$^{-1}$ were due to the -O.H. and N.H. amines groups, respectively. The wavenumber at 3350 cm$^{-1}$ corresponds to the O-H functional group in glucosamine hydrochloride. The N-H and O-H stretching



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**Table 1)** Yield (%) of chitin and glucosamine isolated from shrimp shells.

| Sample | Yield (%) |
|---|---|
| Chitin | 19.90[a] ± 1.02 |
| Glucosamine hydrochloride | 82.52[c] ± 3.63 |
| Glucosamine sulfate sodium chloride | 75.53[b] ± 2.54 |
| Glucosamine sulfate potassium chloride | 82.11[c] ± 3.60 |

Different capital letters (a, b, and v) represent statistically significant differences at ($p < 0.05$).

vibrations were observed at 3345 $cm^{-1}$ and 3275 $cm^{-1}$ in the structure of glucosamine [39]. The $NH_2$ bending vibration was observed at 1540 $cm^{-1}$. Kim et al. [40] reported a similar vibration peak of 1545 attributed to -$NH_2$ [40].

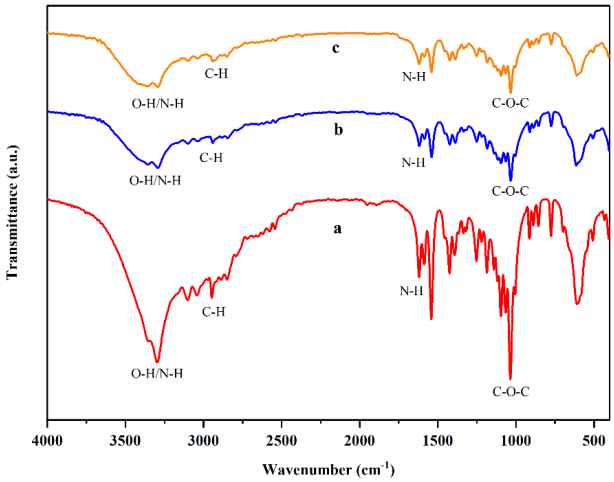


**Figure 2)** FT-IR spectra of (a) glucosamine hydrochloride, (b) glucosamine sulfate potassium chloride, (c) glucosamine sulfate sodium chloride.

## SEM/EDX analysis

SEM images and EDX spectra of (a) G-HCl, (b) GS-Na, and (c) GS-K were obtained to understand the morphology of the surface and confirm the elemental compositions of samples, respectively. As shown in (Figure 3a-c), glucosamine hydrochloride and glucosamine sulfate exhibited polyhedral crystalline structures. It was similar to that noted by Wahab et al. [41] and Al-Hamidi et al. [42], who reported the prismatic shape (polygonal) of glucosamine hydrochloride. The quantitative analysis of glucosamine hydrochloride indicated the presence of carbon (4.87 %), oxygen (42.85%), and chloride (16.28%), which attributed to glucosamine hydrochloride. The analysis showed a peak in the glucosamine hydrochloride, confirming the production of glucosamine hydrochloride (Figure 3a). Moreover, the presence of sulfate (3.44%) and sodium (4.06%) in glucosamine sulfate sodium chloride (Figure 3b) and sulfate (5.52%) and potassium (13.02%) in glucosamine sulfate potassium chloride (Figure 3c) confirmed the isolation of these sulfate salts of glucosamine.

## Differential scanning calorimetry (DSC)

DSC thermogram of G-HCl, GS-Na, and GS-K was measured. The peak temperatures and melting enthalpies (ΔH) of each component are displayed in Figure 4A. Glucosamine hydrochloride showed two endothermic peaks, one at 196.76 ºC, which was attributed to the melting point of glucosamine hydrochloride, and the other peak appeared at 267.35 ºC. The total enthalpy was 508.99 $J.g^{-1}$ and 15.024 $J.g^{-1}$, respectively. A single exothermic peak was exhibited at 384.67 ºC with an enthalpy of 11.661 $J.g^{-1}$ (Figure 4). Three endothermic peaks were appeared at 197.52 ºC (ΔH = 4.517 $J.g^{-1}$), 212.86 ºC (ΔH = 39.03 $J.g^{-1}$) and 227.79 ºC (ΔH = 98.946 $J.g^{-1}$). These melting peaks belong to glucosamine sulfate potassium chloride. The DSC diagram of glucosamine sulfate sodium chloride indicated two endothermic peaks at 223.20 ºC (ΔH = 438.45 $J.g^{-1}$) and 255.88 ºC (ΔH = 1.0326 $J.g^{-1}$). It is followed by an exothermic peak at 393.80 ºC and 7.5273 $J.g^{-1}$ enthalpy. The DSC curve of glucosamine



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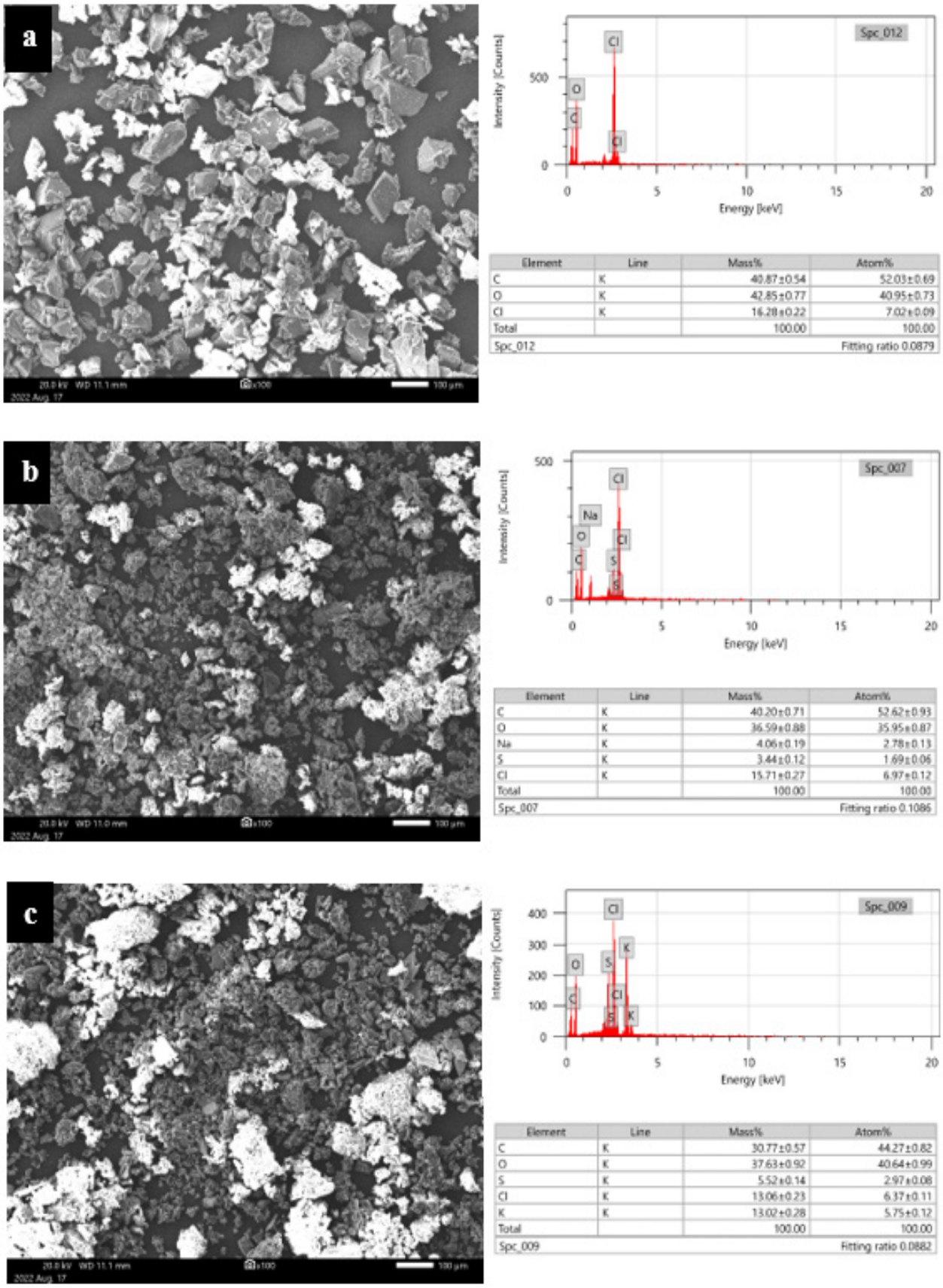


**Figure 3)** SEM image and EDX profile of (a) glucosamine hydrochloride, (b) glucosamine sulfate sodium chloride, and (c) glucosamine sulfate potassium chloride.



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sulfate illustrated a sharp endothermic peak at 218.65 ºC, attributed to the crystalline structure and melting temperature [43,44].

**Thermogravimetric analysis (TGA)**

TGA plots of G-HCl, GS-Na, and GS-K are depicted in Figure 4B. Based on Figure 5a, two strong exothermic peaks are found at 191.26 ºC and 590 ºC. The corresponding weight loss is 55.11 % and 72.86 %, respectively. In the glucosamine sulfate potassium chloride sample, the first mass loss was 35.80 % at around 207.21 ºC, and the second was 82.05 % at around 843.59 ºC. Glucosamine sulfate sodium chloride showed the same trend as other samples. The first weight loss was noticed at 200.24 ºC (41.05 %), and the second was 78.82 % at 790.69 ºC.

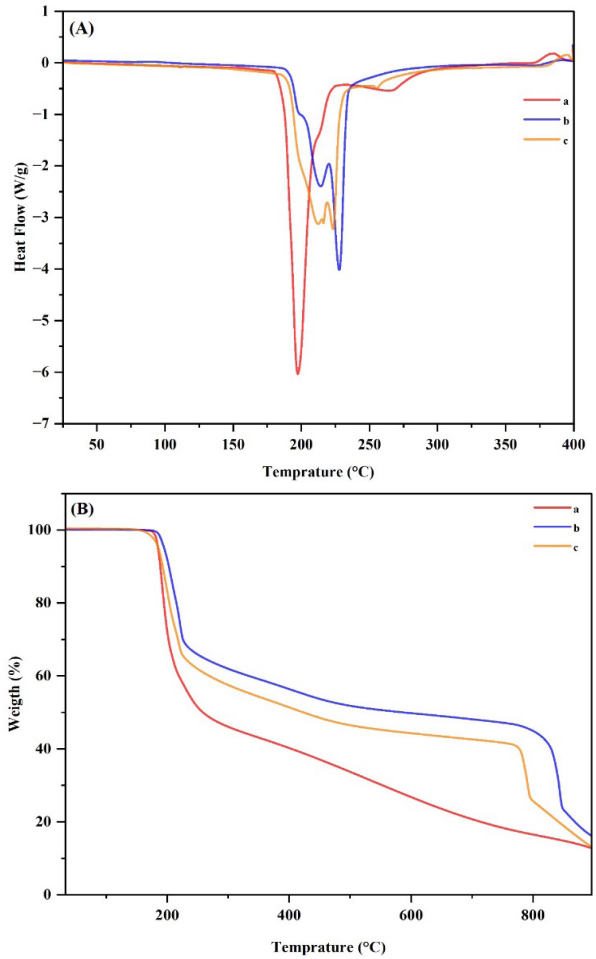


**Figure 4)** (A) DSC and (B) TGA thermograms of (a) glucosamine hydrochloride, (b) glucosamine sulfate potassium chloride, and (c) glucosamine sulfate sodium chloride.

**DPPH radical scavenging activity**

The DPPH free radical (2,2-Diphenyl-1-picrylhydrazyl) is widely performed to characterize the antioxidant capacity of natural compounds [45]. Antioxidant activity of G-HCl, GS-Na, GS-K, and G-S was determined in the concentration range of 25 μg.mL$^{-1}$ to 200 μg.mL$^{-1}$ using DPPH radical scavenging (Figure 5), with ascorbic acid as the standard sample at the same concentration. Generally, DPPH radical scavenging activity increased with increasing concentration. The DPPH free radical abilities of glucosamine hydrochloride were 13.24% and 19.40% at concentrations of 25 μg.mL$^{-1}$ to 200 μg.mL$^{-1}$, respectively. Among the samples, glucosamine standard (22.38%) and glucosamine sulfate potassium chloride (23.32%) indicated higher radical scavenging activities. However, ascorbic acid showed radical scavenging capacity ranging from 75.46% to 88.43% in 25 μg.mL$^{-1}$ and 200 μg.mL$^{-1}$, respectively. This result showed that glucosamine could not reduce the free radical compared with standard ascorbic acid (75.46% to 83.43%) in concentrations ranging from 25 to 200 μg.mL$^{-1}$.

**ABTS radical scavenging activity**

ABTS radical scavenging activity of G-HCl, GS-Na, GS-K, and G-S at various concentrations from 25 to 200 μg.mL$^{-1}$ are presented in Figure 5. Based on these results, glucosamine standard (16.06%) exhibited significant differences at a concentration of 50 μg.mL$^{-1}$ ($p$<0.05). In all samples ranging from 25 to 200 μg.mL$^{-1}$, the greatest radical scavenging activity was for the glucosamine hydrochloride (19.01%) and the least for the glucosamine sulfate potassium chloride (15.51%). As shown in Fig. 6e, ascorbic acid showed 75.46 % and 88.43 % of radical scavenging capacity at the same concentrations of 25 μg.mL$^{-1}$and 200 μg.mL$^{-1}$, respectively. Our present study showed that glucosamine could not inhibit



[ DOI: 10.22034/ECOPERSIA.12.2.6 ]

oxidative damage at 25 to 200 µg.mL$^{-1}$ concentrations.

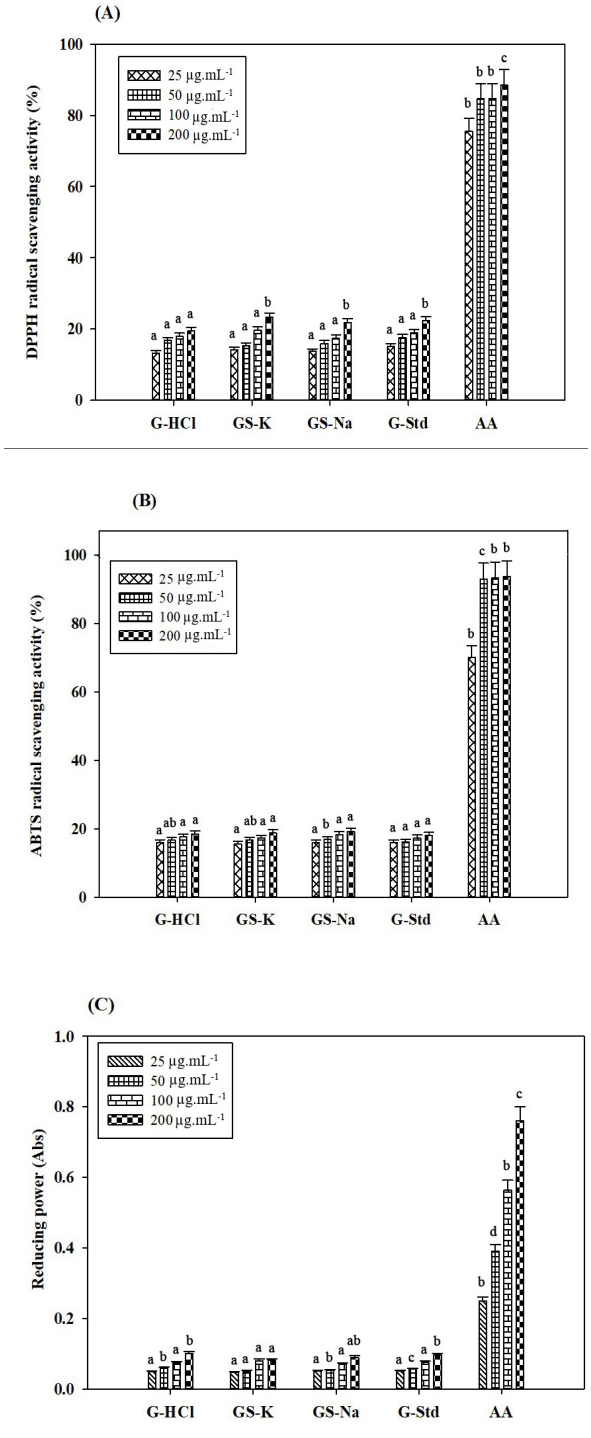


**Figure 5)** (A) DPPH and (B) ABTS radical scavenging activities and (C) ferric reducing power of (a) glucosamine hydrochloride (b) glucosamine sulfate potassium chloride (c) glucosamine sulfate sodium chloride, glucosamine standard (d) and ascorbic acid (e). Different small letters (a, b, and c) represent statistically significant differences at ($p$ < 0.05).

## Ferric reducing power

Ferric reducing ability of G-HCl, GS-Na, GS-K, and G-S are reported in Figure 5. On the contrary, statistically significant differences ($p$<0.05) were seen between samples at the concentration level of 50 µg.mL$^{-1}$. The absorbance of G-HCl, GS-K, G-S, GS-Na, and ascorbic acid were 0.048, 0.046, 0.050, 0.05, and 0.24, which increased to 0.1, 0.08, 0.08, 0.09 and 0.76 at the concentration level of 25 µg.mL$^{-1}$ to 200 µg.mL$^{-1}$, respectively. The result of reducing power showed the least antioxidant capacity of samples compared to the content of ascorbic acid (0.24, 0.39, 0.56, and 0.76) as standard at the concentration of 25 to 200 µg.mL$^{-1}$. Results indicated that the reducing power improved with the increasing concentration of glucosamine.

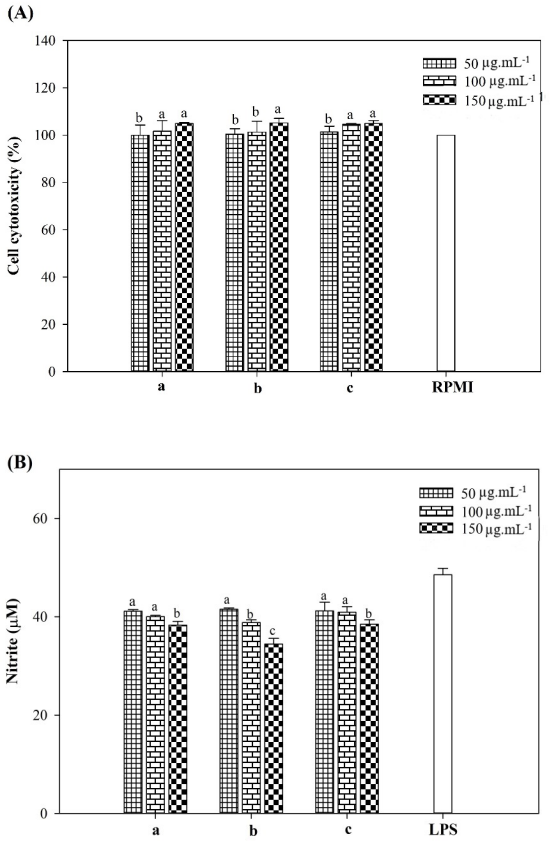


**Figure 6)** RAW264.7 cell (A) cytotoxicity and (B) nitric oxide production under stimulation of (a) glucosamine hydrochloride, (b) glucosamine sulfate sodium chloride, and (c) glucosamine sulfate potassium chloride. Different small letters (a, b, and c) represent statistically significant differences at ($p$ < 0.05).



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**Cytotoxicity and anti-inflammatory activities of glucosamines**

The murine macrophage cell line (RAW264.7) was treated with polysaccharides within the 50–150 μg.mL$^{-1}$ concentration for 18 h to assess potential adverse effects. As illustrated in Figure 6A, no cytotoxicity effects were observed on the growth of RAW264.7 cells when cultured in a medium supplemented with G-HCl, GS-Na, and GS-K. In LPS-induced macrophages, NO production is a typical indicator of conventional inflammatory responses. As depicted in Figure 6B, the presence of 1.0 μg.mL$^{-1}$ of LPS notably triggered NO release in macrophage cells. However, G-HCl, GS-Na, and GS-K exhibited a dose-dependent reduction in NO levels.

## Discussion

The amount of chitin obtained from different sources is believed to be incredibly varied depending on the type of origin and the methodology employed for isolation. For instance, Bertuzzi et al. [37] reported 37% of chitin content isolated from shrimp shells of *Litopenaeus vannamei* using 0.5 M HCL (37%) and 1M NaOH. Kamala et al. [46] reported that the yield of chitin isolated from *Parapenaeopsis stylif*orm shells using 1000 mL of 7 % HCl (w/w) and 1000 mL of 10% NaOH (w/w) was 32%, which was much higher than that obtained in the present research. Trung and Phuong [56] reported that the content of chitin treated with 2% NaOH (w/v) and 4% HCl from shrimp wastes was 9.3% based on a dry basis. Similarly, Mohan et al. [57] reported that the overall yield of chitin isolated from *Litopenaeus vannamei* and *Penaeus monodon* shells was about 20%. The chitin isolated from the exoskeletons of crustaceans is also reported to range between 15 and 40% [58]. By increasing the reaction temperature to 95 ± 1 ºC, chitin was hydrolyzed during the initial time (0-15 min), resulting in a dark brown color, which caused Millard's reaction, which involved amino groups reacting with an aldehyde. The glucosamine sulfate was formed by adding sodium and potassium sulfate to glucosamine hydrochloride. Benavente et al. [47] found that the maximum yield of glucosamine hydrochloride isolated using acid hydrolysis of chitin from shrimp shells was 58%. Bertuzzi et al. [37] reported that the yield of glucosamine isolated from shrimp shells of *Litopenaeus vannamei*, cicada sloughs, and cockroaches were 13.70, 20.46 and 3.28%, respectively. In this study, the yield of G-HCl from shrimp shells was higher than previously reported. The efficiency of glucosamine hydrochloride was positively associated with acid concentration, solid-to-liquid ratio, and reaction time [59].

Generally, it has been stated that there is a direct connection between antioxidant activities and carbohydrate concentrations [55]. Jamialahmadi et al. [53] reported that G-HCl significantly decreased the DPPH radical scavenging activity at various concentrations (5, 10, 20, and 40 mM). Wang et al. [60], Xing et al. [29], and Xing et al. [30] found that ABTS radical scavenging activities of glucosamine hydrochloride and glucosamine sulfate increased dose-dependently with increasing their concentrations. However, numerous studies on glucosamine have reported that the antioxidant activity did not increase significantly with increasing concentrations [55]. Similarly, Gottardi et al. [46] reported that glucosamine hydrochloride did not exhibit any DPPH scavenging activity at a concentration of 0.2 mM. Several factors such as molecular weight, chemical composition, extraction, and separation methods of carbohydrates closely affect their biological activities, especially antioxidant capacity [61,62,63].

The most important response of the immune system to infection and injury, which may characterized by any feeling such



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as pain, heat, and redness, is inflammation [64]. In terms of the inflammatory system, glucosamine not only inhibited the expressions of inducible nitric oxide synthase and IL-1β in lipopolysaccharide-activated microglia and the J774A macrophage cell line in vitro but also demonstrated significant improvements in inflammatory bowel disease and lung inflammation in vivo [65,66,67,68]. According to the current study, all the glucosamine derivatives, including G-HCl, GS-NA, and GS-K, exhibited some extent of anti-inflammatory effects on LPS-induced RAW2647 macrophage cells.

## Conclusion

The chitin and glucosamine products were isolated from the shells of *L. vannamei*. Analysis using HPAEC-PAD, FT-IR, SEM, EDX, DSC, and TGA revealed the successful production of G-HCl, GS-Na, and GS-K. Despite relatively low DPPH and ABTS radical scavenging activities, as well as ferric reducing power, observed for all glucosamine products, they exhibited no cytotoxicity and down-regulated the release of NO in LPS-induced RAW264.7 murine macrophage cells. In conclusion, the results suggest that glucosamine products derived from L. vannamei possess antioxidant and anti-inflammatory properties. Nonetheless, additional investigation is required to evaluate the efficacy of obtained glucosamine in animal models and enhance the bioactivity of glucosamine using chemical and enzymatic means.

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**Author Contribution:** Alika Jafari, leading researcher/data acquisition; Mehdi Tabarsa, conceptualization/supervision; Hossein Naderimanesh, conceptualization/ supervision; Hassan Ahmadi Gavlighi, methodology; SangGuan You, methodology

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