Maillard reaction products of chitosan and glucosamine: antibacterial and antioxidant activity

Le Anh Quoc[1, 2], Dang Van Phu[1], Nguyen Ngoc Duy[1], Nguyen Quoc Hien[1], Ngo Dai Nghiep[2]

[1]*Research and Development Center for Radiation Technology, Vietnam Atomic Energy Institute*
202A, Str. 11, Linh Xuan Ward, Thu duc District, Ho Chi Minh City
[2]*University of Science, Vietnam National University, Ho Chi Minh City Vietnam*
227 Nguyen Van Cu Str., District 5, Ho Chi Minh City
anhquoc1704@gmail.com

Abstract: The investigation of some biological activities was carried out for Maillard reaction products of chitosan and glucosamine (CTS-GA MRPs) prepared by Co-60 gamma irradiation method. A mixture of chitosan (1%) - glucosamine (0.5%) was irradiated with a dose range of 0-100 kGy to induce Maillard reaction for CTS-GA MRPs. The formations of MRPs were confirmed by spectrophotometry and the contents of remaining glucosamine were evaluated by high performance liquid chromatography technique. The antibacterial and antioxidant activities of various CTS-GA MRPs formed at different doses were investigated by direct-contact cell-culture test and $ATBS^{\bullet+}$ free radical scavenging method respectively. The results indicated that CTS-GA MRPs obtained by irradiation at 25 kGy exhibited high antibacterial activity at both pH 5 and 7, while the antioxidant activity of CTS-GA MRPs increased continuously with irradiation dose. CTS-GA MRPs with high antimicrobial and antioxidant properties have a great potential to be applied as a natural preservative for food and cosmetics.

Keywords: *chitosan, glucosamine, Maillard reaction, gamma Co-60, antibacterial, antioxidant*

I. INTRODUCTION

In recent years, owing to the increasing consumer's awareness and concern about the safety of synthetic chemical additives, research on natural alternatives for these additives has attracted considerable attention. The application of several biological materials as natural food additives is one of the most attractive strategies because of their supposed safety. The potent sources of these biological materials are very diverse, including essential oils from plant, enzymes from animals, bacteriocins from microbial sources, organic acids and natural polymers [1]. Among these compounds, chitosan has received considerable interest for commercial applications in medical, agricultural, chemical and food industry. Chitosan, which is composed of D-glucosamine and N-acetyl-D-glucosamine, is a deacetylated derivative of the second most abundant biopolymer – chitin [2]. Chitosan is nontoxic, biocompatible, biodegradable and biofunctional [3]. Some unique biological activities, such as being antibacterial and antioxidative, attract a great deal of attention to chitosan as a potential food preservative of natural origin [4, 5]. In fact, chitosan has been approved as food additive in Japan and Korean since 1983 and 1995, respectively [6, 7]; and in 2001, shrimp-derived chitosan has achived a GRAS (Generally Recognized as Safe) for usage in foods, including meat and poultry, by US Food and Drug Administration [8].

The applications of chitosan for food have been widely reported in many studies, such as in fruit and vegetable [9, 10], seafood [11]; meat and meat products [2, 4, 8, 12, 13]. Unfortunately, the applications of chitosan are limited by its solubility. Chitosan can only dissolve in acidic mediums while in neutral/alkaline mediums, it is precipitated and its biological activities aer reduced as a result. Therefore, several studies have been carried out to improve the solubility and/or the biological activities of chitosan upon chemical and enzymatic modifications, in which chemical modification are generally not preferred in food applications [14].

Maillard reaction, a non-enzymatic browning reaction, is a complex chemical reaction deriving from condensation between a carbonyl group of reducing sugars, aldehydes or ketones, and an amino group of amino acids, proteins or any nitrogenous compounds [13]. Many studies have reported that a myriad of products ~~are~~ formed by Maillard reaction, generally termed Maillard reaction products (MRPs), which mainly contribute to the antioxidant and antibacterial activities [15]. This is a desirable strategy to modify chitosan in order to improve its bioactivities positively. Moreover, there is an increasing scientific interest in carrying out Maillard reaction by irradiation method because by this way, the reaction takes place more rapidly without the temperature control as well as other chemical reagents. Recently, the radiation effects of gamma ray on MRPs formation of chitosan-glucose solution have been investigated to enhance its bioactivities [16]. In addition, a chitosan-glucosamine derivative prepared by heating Maillard reaction has been reported to have a relatively higher antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* as compared with the native chitosan [16]. However, there is no report on the formation of MRPs of chitosan-glucosamine solution upon irradiation as well as the resultant effect on antioxidant and antibacterial activity. The present studies were therefore carried out to investigate the formation of MRPs by gamma irradiation treatment of chitosan and glucosamine solution and the antioxidant and antibacterial activities properties of as-prepared MTPs were also investigated.

II. CONTENT

A. Material and methods

Materials: Chitosan from shrimp shell with the average molecular weight (Mw) of 123.5 kDa and the degree of deacetylation of 93.3 % was supplied by a factory in Vung Tau province, Vietnam. Glucosamine was purchased from Merk (Germany). The *E. coli* ATCC 6538 was provided by Metabolic Biology Laboratory, University of Science, Ho Chi Minh City; then cultivated and preserved at Biology Laboratory, VINAGAMMA, Ho Chi Minh City. The Luria- Bertani medium and agar plates used for bacteria incubation were purchased from Himedia, India. Ultra-pure ABTS diammonium salt and potassium ferricyanide were products from Sigma-Aldrich. Other chemicals such as: lactic acid, H_2O_2,… are used in analytical grade. Distilled water is used for all experiments.

Preparation of chitosan-glucosamine MRPs

The preparation of chitosan-glucosamine MRPs solutions was carried out according to the method of Rao et al. (2011) with some modification [16]. A 2% solution of chitosan in acetic acid (1%) was prepared. Similarly, various solutions of glucosamine in distilled water were prepared with different contents of 1, 2 and 4 % respectively. The chitosan solutions were mixed to these glucosamine solutions with the ration 1:1 (v:v) separately in order to obtain three mixture solutions, namely A solution: chitosan 1% - glucosamine 0.5%; B solution: chitosan 1% - glucosamine 1% and C solution chitosan 1% - glucosamine 2%. All solutions were exposed to γ-irradiation with different doses in the range of 0–100 kGy by a Gamma-cell 5000 (BRIT, Mumbai, India) supplying a dose rate of 2.2 kGy/h.

Spectrophotometric analyses

The irradiated solutions were characterized by spectrophotometric analyses described by Chawla et al. (2009) [18]. The as-prepared solutions were appropriately diluted and the absorbance was measured at 284 nm (early Maillard reaction products) and 420 nm (late Maillard reaction products) for determining UV absorbance and browning intensity, respectively by a UV–vis spectrophotometer, Jasco-V630, Japan.

Determination of glucosamine content

The glucosamine content of irradiated solutions were determined by high performance liquid chromatography (HPLC) method according to AOAC 2012 (2005.01) standard at Binh Duong Quality Control Centre, Vietnam. Maillard reaction efficiency was expressed as the ratio of reacted glucosamine to the total added glucosamine by the formula:

$$\text{Maillard reaction efficiency (\%)} = (M_0 - M_t) \times 100/M_0 \quad (1)$$

Where M_0 and M_t are glucosamine contents of the CTS-GA solution before and after irradiated, respectively.

Determination of antioxidant activity

Antioxidant activities of glucosamine solution and the irradiated solutions were determined by $ATBS^{\bullet+}$ radical scavenging ability assay described by Zhai et al. [19] and Chen et al. [20] with slight modification. Briefly, 0.6 ml of samples was thoroughly mixed with 1 ml $ATBS^{\bullet+}$ radical solution to obtain the desired concentrations. $ATBS^{\bullet+}$ radical solution was prepared by mixing 7.4 mM ABTS and 2.6 mM $K_2S_2O_8$ in aqueous solution with the same volume and kept in the dark for 16h at room temperature, and then diluted by water to reach the optical of density 1 ± 0.1 at the wavelength of 734 nm (OD_{734}) on a UV-vis spectrophotometer. The 1 ml ABTS solution (without $K_2S_2O_8$) diluted with water was also added 0.6 ml sample with the same concentration for preparation of the blank samples. The OD_{734} measuring was carried out in triplicate for each sample and the percentage of $ATBS^{\bullet+}$ radical scavenging ability was calculated as the following equation:

$$ATBS^{\bullet+} \text{ radical scavenging ability (\%)} = (A_C - A_S) \times 100/A_C \quad (2)$$

Where A_C is the OD_{734} of the control ($ATBS^{\bullet+}$ radical solution and water) and the A_S is the OD_{734} of $ATBS^{\bullet+}$ radical solution and tested solutions.

Evaluation of antibacterial activity

The antibacterial activity of chitosan-glucosamine (CTS-GA) MRPs prepared at different irradiation doses in the range of 0-100 kGy was investigated against *Escherichia coli 6538* in both qualitative and quantitative tests.

In qualitative test, the agar well diffusion method was used as described by Balouiri et al. [21]. The LB agar plates, after being spread by *E. coli* (~ 10^4 CFU/ml) on the surface, were punched aseptically with a sterile tip to form wells with a diameter of 6 mm. 100 µl of CTS-GA MRPs prepared with different irradiation doses of 0-100 kGy were introduced to the wells respectively. Then the plates were incubated overnight at 37ºC and monitored colony formation. The glucosamine solution was also tested by this method as the control.

In quantitative test, the antibacterial activity of CTS-GA MRPs against *E. coli* was investigated at pH 5 and 7. Briefly, 1 ml CTS-GA MRPs solutions were simultaneously added into 19 ml *E. coli* suspensions (10^7 CFU/ml), in which the pH was already adjusted to 5 and 7 by lactic acid 0.5 % and/or NH_4OH 5% solution. Then the mixtures were shaken at 150 rpm for 4 hours and subsequently determined the survival cell density by spread plate technique. The control sample only containing bacteria suspension and water was carried out in parallel. The antimicrobial activity of the CTS-GA MRPs was expressed by the reduction of bacteria density (log CFU/ml) in the testing mixture in comparison with the control sample.

B. Results and discussion

Formation of CTS-GA MRPs

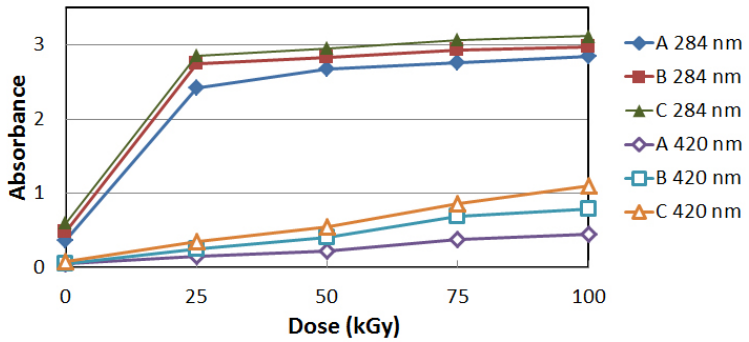

Fig. 1. UV absorbance (284 nm) and browning (420 nm) of irradiated CTS-GA solutions at various irradiation doses (A: CTS 1% - GA 0.5%; B: CTS 1% - GA 1% and C: CTS 1% - GA 2 %)

There was a change in visual color of the CTS-GA solutions from colorless to dark brown during the irradiation process. Moreover, an increase in UV absorbance and browning intensity of CTS-GA solutions as the effect of the irradiation dose was also observed by spectrophotometric analyses (Fig. 1). The same results were recorded in other studies where the protein/sugar solutions were treated by heating [18] or irradiating [16]. In addition, although the CTS:GA ratio was different, the various solutions had a similar change in UV absorbance and browning intensity. The 284 nm absorbance increased dramatically in the dose range of 0-25 and then nearly steady up to the dose of 100 kGy while the 420 nm absorbance increased regularly with the increasing irradiation dose. In Maillard reaction, the UV absorbance intermediate compounds were developed prior to the generation of brown pigments. Therefore the results of spectrophotometric analyses indicate that during the irradiation process, the MRPs were formed, in which the formation of early MRPs were almost saturated at the dose of 25 kGy, while the late MRPs were produced continuously along with the dose up to 100 kGy.

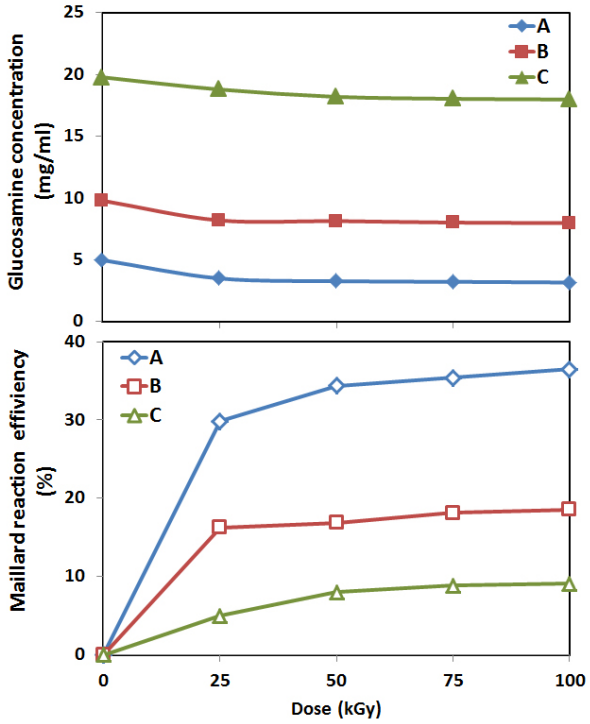

Fig. 2. The glucosamine concentration and Maillard reaction efficiency of CTA-GA solutions versus irradiation dose (A: CTS 1% - GA 0.5%; B: CTS 1% - GA 1% and C: CTS 1% - GA 2 %)

The glucosamine concentration and the Maillard reaction efficiency of CTA-GA solutions prepared at different irradiation doses were described in Fig. 2. The results show

that the glucosamine concentration of the CTS-GA solution decreased dramatically in the dose range of 0-25 kGy and then almost steady up to the dose of 100 kGy. The presence of glucosamine in the solution after the irradiation process meant that the initial glucosamine content was redundant for the reaction in all tested CTS-GA solutions. Moreover, at the dose of 100 kGy, the decrease of glucosamine concentration in the various CTA-GA solutions was nearly equal and of about 1.8 mg/ml (data not shown). This could be the suitable concentration of glucosamine for reacting with the 1% chitosan solution.

In addition, the results in Fig. 2 also showed that in all solutions, Maillard reaction efficiency increased along with the irradiation dose, in which the highest rate of the increase was belong to the dose range of 0-25 kGy. This tendency was similar to the increasing of UV absorbance. Therefore the result suggested that the as-calculated efficiency could be represented for the formation of the early MRPs because during the irradiation process, only early reactions consumed glucosamine and caused the decrease of its concentration in the solution, while the late reactions just polymerized the intermediates, formed colored polymers [18, 22] and did not affect the glucosamine concentration.

Determination of antioxidant activity

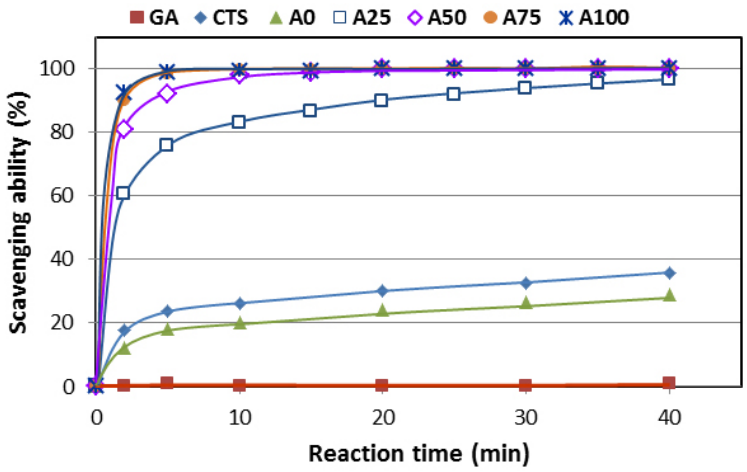

Fig. 3. The relationship of $ATBS^{\bullet+}$ radical scavenging ability versus reaction time (GA: glucosamine; CTS: chitosan; A0, A25, A50 and A100 were the A solutions irradiated with the dose of 0, 25, 50, 75 and 100 kGy respectively)

The results presented in Fig. 3 indicated that the glucosamine solution (0.5%) hardly exhibited $ATBS^{\bullet+}$ radical scavenging ability while the inverse was true for chitosan-containing solutions. Moreover, the $ATBS^{\bullet+}$ radical scavenging ability of chitosan solution was higher than CTS-GA solution (A0 sample). This finding was suggested to be due to the obstacle of glucosamine over chitosan on scavenging $ATBS^{\bullet+}$ radical. Furthermore, the irradiated CTS-GA solutions manifested the high capability of scavenging $ATBS^{\bullet+}$ radical in dependence on the irradiation dose and reaction time. In other words, the higher irradiation dose and/or reaction time, the higher $ATBS^{\bullet+}$ radical scavenging capacity. The formation of antioxidant compound by heating sugar-amino solution has been reported [23]. This result indicated that the antioxidant compounds formed upon irradiation of chitosan-glucosamine solution had significant antioxidant potential. In addition, the dose-dependent antioxidant activity of irradiation treating chitosan-glucose solution has also been recorded [16]. As the above discussion, during irradiation treatment, the early MRPs was almost saturated at the dose of 25 kGy, while the late MRPs were produced continuously up to 100 kGy, so this results suggested that the formation of antioxidant compounds ~~were~~ mainly taken place at the late stage of Maillard reaction.

Evaluation of antibacterial activity

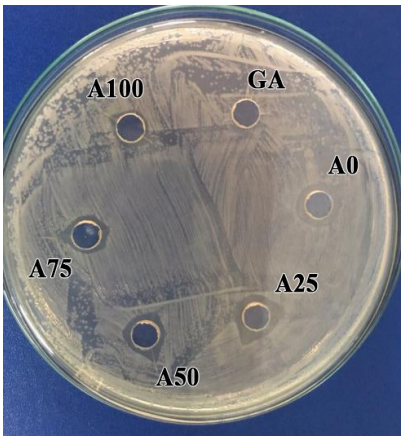

Fig. 4. The result of agar well diffusion test (GA: glucosamine; A0, A25, A50 and A100 were the A solutions irradiated with the dose of 0, 25, 50, 75 and 100 kGy respectively)

In Fig. 4, the A solutions prepared at different irradiation doses were able to form the inhibition zone against *E. coli*; unlike the GA sample. This meant that glucosamine did not exhibit the antibacterial activity in contrast to other A samples. Interestingly, around the well of A0 sample (a CTA-GA solution without irradiation), the presence of the inhibition zone indicated that the antibacterial activity of this solution was due to the role of chitosan. The antibacterial ability of the samples could be primarily compared through the diameters of their inhibition zones formed on the plate [21], therefore the result indicated that the antibacterial activity decreased obviously in A25, A50, A75 and A100 sample respectively.

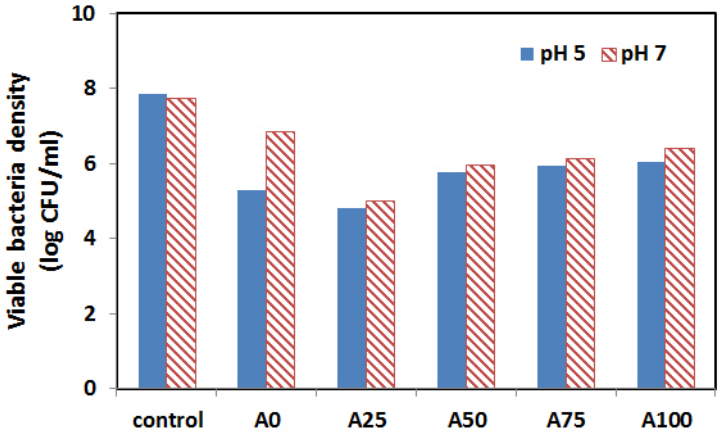

Fig. 5. Viable bacteria density of the suspension after exposing time (A0, A25, A50 and A100 were the A solutions irradiated with the dose of 0, 25, 50, 75 and 100 kGy respectively)

Modification of chitosan via Maillard reaction has been widely studied and it is suggested that MRPs produced from chitosan-sugar model system have been associated with the formation of compounds with high antibacterial [16, 22, 24]. However, there is no information about the influence of irradiation dose on the information of these compounds. Thus, in this study the antibacterial activities of MPRs prepared with different doses were examined and further compared with chitosan. In more detail, after the exposing time, the bacterial cell density of the suspensions at pH 5 and 7 was determined and described in Fig. 5. The result revealed that the antibacterial activity of A0 sample was affected deeply by the pH value, namely been high at pH 5 and low at pH 7. As above discussion, the antibacterial activity of A0 sample was mainly contributed by chitosan, which was precipitated and reduced its bioactivity at neutral or alkaline solution, hence the antibacterial activity of A0 sample at pH 5 was greater than at pH 7. This finding is totally in concurrence with other

studies where the pH-dependent antibacterial activity of chitosan has been reported [17, 25]. In addition, the obtained results also indicated that all testing samples had the lower bacterial cell density in comparison with the control, this meant that these samples exhibited the effective antibacterial activity against *E. coli* at both pH 5 and 7. The lower viable bacteria density represented the stronger antibacterial activity. Therefore at pH 5 and 7, the antibacterial activity of irradiated samples decreased along with the increasing dose and A25 was the most antibacterial sample. This record completely matched with the results of agar well diffusion test above. Furthermore, the higher antibacterial activity of chitosan-glucosamine derivatives prepared by heat-induced Maillard reaction compared to acid-soluble chitosan was also recorded in the study of Chung et al. (2005). In addition, because during the irradiation treatment, the early MRPs were created prior to the formation of late MRPs so the results above suggested that the antibacterial activities of irradiated solutions were probably due to the role of early MRPs. Interestingly, the antibacterial activities of MRPs in irradiated solutions were maintained at high level at both pH 5 and 7. Hence, this result is one of the most obvious demonstrations of Maillard reaction**'s** effectiveness in chitosan modification strategies.

III. CONCLUSION

CTS-GA MRPs were efficiently synthesized by the Maillard reaction through gamma Co-60 irradiation technique. Other ionizing radiations, such as electron beam, can also be successfully employed to induce Maillard reaction in CTS-AG solution. The results of this study demonstrated that the suitable concentration of glucosamine to take part in Maillard reaction was much lower than chitosan solution, and as-prepared CTS-GA MRPs exhibited high antioxidant activity and strong antibacterial activity at both pH 5 and 7. These findings indicated that CTS-GA MRPs had a great potential as a natural product for synthesis additive replacement. Further studies are necessary to elucidate the mechanism of compounds formed during the irradiation treatment and their identification as well as the real application of these products for food or cosmetic.

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Sản phẩm phản ứng Maillard của chitosan và glucosamine: hoạt tính kháng khuẩn và kháng oxi hóa

Lê Anh Quốc[1], Đặng Văn Phú[1], Nguyễn Ngọc Duy[1], Nguyễn Quốc Hiến[1], Ngô Đại Nghiệp[2]

[1]Trung tâm Nghiên cứu và Triển khai Công nghệ Bức Xạ, Viện Năng lượng Nguyên tử Việt Nam
202A, Đường 11, Phường Linh Xuân, Quận Thủ Đức, TP Hồ Chí Minh
[2]Trường Đại học Khoa học Tự nhiên, Đại học Quốc gia Thành phố Hồ Chí Minh
227 Nguyễn Văn Cừ, Phường 4, Quận 5, TP Hồ Chí Minh
anhquoc1704@gmail.com

Tóm tắt: Một số hoạt tính sinh học của sản phẩm phản ứng Maillard giữa chitosan và glucosamin (CTS-GA MRPs) chế tạo bằng phương pháp chiếu xạ gamma Co-60 đucợ tiến hành nghiên cứu. Hỗn hợp chitosan (1%) - glucosamine (0,5%) được chiếu xạ với khoảng liều là 0-100 kGy để thực hiện phản ứng Maillard. Sự hình thành của MRPs sau khi chiếu xạ được xác định bằng phương pháp phân tích quang phổ và hàm lượng glucosamin còn lại trong hỗn hợp cũng được xác định bằng kỹ thuật sắc ký lỏng hiệu năng cao. Ngoài ra, các CTS-GA MRPs chế tạo ở các liều xạ khác nhau còn được khảo sát hoạt tính kháng vi khuẩn *E. coli* và kháng oxi hóa lần lượt bằng phương pháp tiếp xúc trực tiếp và bắt gốc tự do $ATBS^{\bullet+}$. Kết quả cho thấy, CTS-GA MRPs 25 kGy thể hiện hoạt tính kháng khuẩn mạnh ở cả pH 5 và 7, trong khi hoạt tính kháng oxi hóa của các CTS-GA MRPs tăng theo liều xạ. Các CTS-GA MRPs với khả năng kháng khuẩn và chống oxi hóa mạnh rất có tiềm năng ứng dụng làm chất bảo quản tự nhiên dùng trong thực phẩm và mỹ phẩm.

Từ khóa: *chitosan, glucosamine, phản ứng Maillaerd, gamma Co-60, kháng khuẩn, kháng oxi hóa*