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Physicochemical Properties and Shelf Life of Button Mushroom under Essential Oils with Chitosan Coating Treatment

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ABSTRACT

Agaricus bisporus is the most produced mushroom in the world due to its unique taste and valuable nutritional properties. For this purpose, a study in the form of a completely randomized design with 5 treatments and 3 repetitions was conducted, which includes (control) (chitosan 2%) (chitosan 2% + cinnamon essence 2%) (chitosan 2% + angelica essence with a concentration of 200 ppm) (chitosan 2% + cinnamon essence 2% + angelica essence with a concentration of 200 ppm). The properties of *Agaricus bisporus* were evaluated on days 0, 7, 14, and 21. The results indicated 7 and 14 days after storage, chitosan casing as well as chitosan essence + angelica and cinnamon essence increased the phenolic acid content compared to the control and zero days after storage. On the 7th, 14th, and 21st days after storage, chitosan + angelica treatment showed the highest weight loss. The lowest weight loss was observed 21 days after storage in control and chitosan treatment. The highest number of soluble solids was observed 7 days after storage in chitosan + angelica essence and chitosan + cinnamon essence. The highest color index L* was observed at zero days after storage in the control treatment. The highest color index a* was observed on the 14th day of storage in the chitosan + angelica + cinnamon treatment and on the 7th day of storage in the chitosan treatment. The lowest color index a* was obtained on the 14th day of storage in the chitosan + cinnamon treatment and on the 7th day of storage in the chitosan + angelica +

cinnamon and chitosan + cinnamon treatments. The highest b* color index was observed in the control treatment at 21 days after storage. In general, treatments showed different results on biochemical properties and post-harvest shelf life of *Agaricus bisporus.*

***Key Words*:** Chitosan, Essential oil, Edible mushroom, Soluble solids

INTRODUCTION

The mushroom with the scientific name *Agaricus bisporus*, is the most widely produced edible mushroom in the world (Zhang *et al*., 2020). The browning of an enzyme in fruits and vegetables causes adverse quality changes during handling, packaging, and storage, moreover a way to prevent and control these contaminations is always essential (Hosseinzadeh *et al*., 2015). Post-harvest treatments are used to maintain the quality or improve the appearance of horticultural products. To increase life after harvest, suitable methods and compounds should be chosen that do not harm the quality of the fruiting body and are also useful for the consumer (Song *et al*., 2018). One of these ways is to use the essential oils of medicinal plants. Plant essential oils prevent enzymatic browning due to their antioxidant role. In addition, the antimicrobial properties of some essential oils have also been proven. Therefore, the use of these materials has a great effect on increasing the shelf life of horticultural products and food (Qu *et al*., 2020). Due to the concerns about the use of chemical compounds and their harm, many studies have been conducted regarding the effective compounds of different types of medicinal plants and the effect of these compounds on microorganisms (Nasiri *et al*., 2018). Due to the perishability of fruits and vegetables, it is very necessary to use new technologies to prevent waste after harvesting these products (Montero Pardo *et al*., 2011).

Plant essential oils have been proposed as a new method to control post-harvest diseases and increase the quality and length of the storage period (Qin *et al*., 2015). Therefore, according to the increasing limitations of the use of antimicrobial chemicals, it seems that natural essential oils and volatile oils are better sources of antimicrobials to preserve edibles (Fakhreddin *et al*., 2013; Khazraei *et al*., 2016). Storage is also one of the most important post-harvest processes because improper storage conditions are one of the important causes of quantitative and qualitative damage to products (Tabatabae koloor, 2012). Low temperature is used as one of the most effective methods to increase the storage life of agricultural products, therefore, many physiological and anatomical changes are caused by post-harvest damage in the product and fruit tissue (Han *et al*., 2006). The research was conducted to investigate the effect of cinnamon and angelica oil essential oils in combination with chitosan polysaccharide coating under the conditions of packaging type and temperature conditions on the shelf life and some qualitative and biochemical properties of the mushroom.

MATERIALS AND METHODS

The current research was carried out in 2021 at Isfahan Islamic Azad University (Khorasgan). To investigate the effect of chitosan casing along with angelica and cinnamon essences on the quality and shelf life of edible mushrooms, in the form of a completely randomized design with 5 treatments was evaluated including (control) (chitosan 2%) (chitosan 2% + cinnamon essences 2%) (Chitosan 2% + angelica essences with a concentration of 200 ppm) (chitosan 2% + cinnamon essences 2% + angelica essences with a concentration of 200 ppm).

To carry out the research, mushrooms were purchased and transported to the laboratory one hour after harvesting. Then, until the implementation of the project, to maintain the humidity of the fruiting body, the mushroom containers were covered with polyvinyl chloride (PVC). For chitosan-coated treatments, a 2% (w/v) chitosan (Sigma-Aldrich medium molecular weight) solution in 1% (v/v) acetic acid (Sigma-Aldrich) was prepared. Then, 0.75% glycerol (Sigma-Aldrich) was added to it as a plasticizer. In the next step, the pH of the solution was adjusted to 5 with 1 normal soda and finally, 0.2% (v/v) of polysorbate was added to the prepared solution. After preparing the chitosan solution, the samples were immersed in the prepared solution for 1.5 minutes (Tajik *et al*., 2011). Regarding the treatments in which essence is used, to distribute the essence evenly and completely in the chitosan solution, first, the desired essence was mixed with Tween 80 and then it was added to the chitosan solution (Mojaddar Langroodi and Tajik, 2017).

Angelica essence was purchased and prepared and used at a concentration of 200 ppm using Tween 80 as an emulsifier in the aqueous medium. To prepare the casing with cinnamon essence with a concentration of 2% (volume/volume), 2 ml of essence was dissolved with 1 g of Tween 80 and added to the emulsion. The treatment was done by rapid immersion for 2 minutes. After applying all the treatments on the samples and after drying the samples at room temperature, they were placed in special 100-g packaging containers (with normal cellophane cover) and kept in the refrigerator at 4 ℃ for 20 days. Then on days 0, 7, 14, and 21, sampling was done to perform sensory and biochemical tests. To identify the constituents of the essence, a gas chromatography device coupled with a mass spectrometer (GC/MS) including the Agilent 5975 C mass detector was used with an electron ionization source coupled with the Agilent 7890 gas chromatography device, which uses a 30-meter-long HP-5MS column and the diameter of 0.25 mm, in which the thickness of the stationary phase layer was 0.25 μm.

The amount of weight loss

For this purpose, the fruiting body of the harvested mushroom was weighed immediately after packaging using a digital scale with an accuracy of 0.001 g. At the end of the experiment, their weight was measured again and the amount of weight loss was calculated. In other words, the weight loss in the mushroom samples is due to the weight loss compared

to the initial weight, which was expressed as a percentage according to equation (1) (Zhu *et al*., 2019).

equation (1): Percentage of weight loss = $\frac{Secondary\ weight - primary\ weight}{primary\ weight} \times 100$

Phenol content

Total phenolic content was determined according to Folin- Ciocalteu's method (Singleton and Rossi 1965) with modifi-cations. One gram of frozen tissue was homogenized at 4 °Cwith 5 mL of ice-cold methanol, centrifuged, and filtered, and the filtrate was diluted with 5 mL of distilled water. A sample of 150 µL was transferred in a test tube and added 750 µLFolin-Ciocalteu's phenol reagent and 600 µL sodium carbon- ate solution (7.5 %, w/v); the tube was vortexed and incubated at 25 °C for 1 h, after which the absorbance was measured at 760 nm using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., Beijing, China). Gallic acid was used as the calibration standard, and the data were expressed as gallic acid equivalents in mg g−1 fresh weight (FW) (Ding *et al*, 2016).

Evaluation of the color of mushroom samples

To evaluate the colorimetric test, the color of the samples was evaluated using a special box method. In this method, the RAL standard color cards were first photographed under the same conditions, and then the samples were photographed under the same conditions. Then, with the help of Adobe Photoshop version 8 software, the photos were analyzed and the RGB indicators were extracted. A standard chart was drawn based on the RGB index of RAL color cards and the RGB index of the photo was calibrated with it. Finally, the RGB index was calibrated and the samples were converted to the Lab index. To investigate the number of changes made in the color properties of food during different essential oil treatments, the term parameters or color indices were used (Saxena *et al*., 2010).

L = brightness level, from black (0) to white (100)

a = amount of redness, from green (negative values, -60) to red (positive values, +60)

b = the degree of yellowness, from blue (negative values, -60) to yellow (positive values, +60), indicates the degree of color purity, brightness, or color intensity (angles from 0 to 360 degrees indicate red color and angles from 90, 180 and 270 degrees represent yellow, green and blue colors respectively) (Saxena *et al*., 2010).

Soluble solids

The number of dissolved solids was measured using a digital refractometer and expressed as a Brix percentage. First, the refractometer was calibrated with distilled water, and then the strained extract of the tested samples was placed on the device (a few drops). Then the number shown was considered as the Brix of the sample (Varasteh and Zamani, 2019).

Statistical analysis of data

Finally, the resulting data were analyzed using SAS statistical software, and the averages were compared by Duncan's multi-range test at a probability level of 5%. Excel 2018 software was also used to draw graphs.

RESULTS AND DISCUSSION

Weight Loss

The results of variance analysis of the data showed that the effect of time, treatment, and the double effect of time × treatment were significant at the 1% probability level on the amount of weight loss of the mushroom (Table 1). According to the average comparison results of data, the double effect of time × treatment was significant at the 5% level of Duncan's test on the weight loss of the mushroom. So, on the 7th, 14th, and 21st days of storage, chitosan + angelica treatment showed the highest weight loss with 7.84%, 7.19%, and 6.81%, respectively. The lowest amount of weight loss was also shown on the 21st day of storage in the control and chitosan treatments with 1.51% and 2.11%, respectively. There was no significant statistical difference between some treatments at the 5% level of Duncan's test.

Table 1.The results of variance analysis of the effect of storage time and treatment on the amount of mushroom weight loss

Source of variations	Degree of freedom	Mean square
		Weight loss
Time	2	3.35**
Treatment	4	46.09**
Time × Treatment	8	0.22**
Error	30	0.003
CV (%)		1.15

**: Significant at the statistical level of 1 %

The results of this research indicated that chitosan + angelica treatment showed the highest amount of weight loss in the investigated days. The lowest weight loss rate was shown on the 21st day of storage in control and chitosan treatments. In addition, by increasing the storage time to 21 days, the weight loss rate of the mushroom samples showed a significant increase.

The weight loss of samples during the storage period is due to the difference in water vapor pressure between the samples and the surrounding environment, which causes interstitial water to escape into the space around the sample. This phenomenon is due to the thin epidermal structure of the mushroom cap, which cannot prevent the rapid removal of water

from tissue surfaces. Also, fruit weight loss is a result of water loss from the surface of the fruiting body, and if the weight loss in harvested mushrooms reaches 5-6%, the overall acceptance of the product will be damaged (Karimirad *et al*., 2020). Based on this, casings that retain product water more than other castings have a positive effect on increasing the shelf life of the product. Nasiri *et al*. (2018) stated that all samples of *Agaricus* mushrooms experienced weight loss during 16 days of storage.

The lower weight loss of chitosan-coated mushrooms on day 21 of storage may be due to the properties of the casing as a semi-permeable barrier against carbon dioxide, oxygen, moisture, and solute movement (Rok *et al*., 2020). The role of chitosan in reducing fruit weight can be attributed to its polycationic property. This polycationic casting, by breaking into polymer pieces and re-forming the polymer chain, causes the formation of a gel-like surface coating film (Nasiri *et al*., 2018).

Phenolic acid

The results of variance analysis of the data showed that the effect of time, treatment, and the double effect of time × treatment were significant at the 1% probability level on the amount of phenolic acid in the mushroom (Table 2). According to the average comparison results, the double effect of time × treatment was significant at the 5% level of Duncan's test on the amount of phenolic acid in the mushroom. So, the highest amount of phenolic acid was obtained on the 7th day of storage under the treatment of chitosan coating + angelica essence with the amount of 387.04 mg/kg. On the 7th and 14th days of storage, the application of chitosan coating alone and chitosan enriched with angelica and cinnamon essences increased the amount of phenolic acid compared to the control group and day zero samples. On the 21st day of storage, the amount of phenolic acid under the studied treatments showed a significant decrease compared to days 0, 7, and 14. Among the investigated treatments, the lowest amount of phenolic acid was obtained in the chitosan treatment containing angelica and cinnamon essence with the amount of 146.79 mg/kg. No statistically significant difference was observed between some treatments at the 5% level of Duncan's test.

The results of this research showed that chitosan casing treatment + angelica essence increased the amount of phenolic acid on the 7th day of storage. On the 7th and 14th days of storage, the application of chitosan casing alone and chitosan enriched with angelica and cinnamon essences increased the amount of phenolic acid compared to the control group and day zero samples. On the 21st day of storage, the amount of phenolic acid under the investigated treatments showed a significant decrease compared to days 0, 7, and 14. The changes in phenolic compounds in the post-harvest stage are different depending on the treatment and storage conditions. The higher amount of total phenol in the coated samples can be related to the increase in antioxidant capacity (Vidhan *et al*., 2010). Higher levels of total phenol in coated samples may be due to lower intracellular oxygen concentration and reduced respiration rate (Mirshekari *et al*., 2019).

One of the main reasons for the reduction of phenolic compounds on the 21st day of storage is enzyme activity, some enzymes use phenolic compounds as an intermediate

compound to reduce free radicals to deal with the low storage temperature, which causes a decrease in the amount phenolic compounds (Anjum *et al*., 2019).

The results of this research on the increase of phenolic compounds under the influence of chitosan + angelica essence treatment are consistent with the results of Karimirad *et al*.'s research (2018) on mushrooms under the influence of the chitosan + orange flower essence treatment and Khojah *et al*.'s research (2021) under the effect of chitosan casing containing titanium oxide nanoparticles. In Loquat (a Japanese fruit), with increasing storage time, the amount of total phenol showed a significant decrease, which is consistent with the results of this study (Meighani and Hashempour, 2021).

Color Index L*

The results of variance analysis of the data showed that the effect of time, treatment, and the double effect of time × treatment were significant at the probability level of 1% on the color index L* in the mushroom (Table 2). According to the average comparison results of data, the double effect of time × treatment was significant at the 5% level of Duncan's test on the color index L* of the mushroom. So, the highest L* color index was observed on day zero of storage in the control treatment at 80.83. With the increase in storage time and under the studied treatments, the L* color index showed a significant decrease. The lowest L* color index was observed on the 7th and 14th days of storage in chitosan + cinnamon treatment. There was no statistically significant difference between some of the treatments at the 5% level of Duncan's test.

Color Index a*

The results of variance analysis of the data showed that the effect of time, treatment, and the double effect of time × treatment were significant at the 1% probability level on the color index a* in the mushroom (Table 2). The average comparison results of data showed that the double effect of time × treatment was significant at the 5% level of Duncan's test on the color index a*. So, the highest color index a* was observed on day 14 of storage in chitosan + angelica + cinnamon and chitosan treatment and on day 7 of storage in chitosan treatment with 26.47, 25.53, and 25.35, respectively. By increasing the storage time to 21 days and under the influence of the studied treatments, the color index a* showed a significant decrease. The lowest color index a* was obtained on the 14th day of storage in the chitosan + cinnamon treatment with 1.92 and on the 7th day of storage in the chitosan + angelica + cinnamon and chitosan + cinnamon treatments with 2.47 and 2.82, respectively. No statistically significant difference was observed between some treatments at the 5% level of Duncan's test.

Color Index b*

The results of variance analysis of the data showed that the effect of time was significant at the 1% probability level on the b* color index, while the effect of treatment and the double effect of time × treatment were significant at the 5% probability level on this trait (Table 2). The average comparison results showed that the double effect of time × treatment was significant at the 5% level of Duncan's test on the b* color index. So, the highest b* color index was observed on the 21st day of storage in the control treatment with a rate of 41.78. By increasing the storage time in the control treatment, the b* color index also showed a significant increase. Under the effect of chitosan + cinnamon treatment on the 7th and 14th day of storage and chitosan + angelica + cinnamon treatment on the 7th day of storage, the lowest color index b* was observed. However, no statistically significant difference was observed between some treatments at the 5% level of Duncan's test.

The color indices L*, a*, and b* respectively express the brightness, the degree of redness, and the degree of yellowness of the fruiting body, which is related to the presence of anthocyanin and flavonoid pigments in the fruit. The a* index value indicates the color changes from green to red. With the increase in storage time and under the studied treatments, the L* color index showed a significant decrease. The lowest L* color index was observed on the 7th and 14th days of storage in the chitosan + cinnamon treatment. The highest color index a* was observed on day 14 of storage in chitosan + angelica + cinnamon and chitosan treatment and on day 7 of storage in chitosan treatment. By increasing the storage time to 21 days and under the influence of the studied treatments, the color index a* showed a significant decrease. The lowest color index a* was obtained on the 14th day of storage in the chitosan + cinnamon treatment and on the 7th day of storage in the chitosan + angelica + cinnamon and chitosan + cinnamon treatments. The highest color index b* was observed on the 21st day of storage in the control treatment. By increasing the storage time in the control treatment, the b* color index also showed a significant increase. Under the effect of chitosan + cinnamon treatment on the 7th and 14th day of storage and chitosan + angelica + cinnamon treatment on the 7th day of storage, the lowest color index b* was observed.

The color change is one of the major problems in agricultural products, which can cause pink, red, green, or blue color (Hu *et al*., 2015). When the product suffers tissue damage during processing, its color changes due to enzymatic and chemical reactions. The use of edible casings reduces the process of degradation of pigments and the formation of pheophytin in the tissue (Khan *et al*., 2015).

The parameter b* in the CIElab color system is the color component with a range of changes from blue to yellow, and its increasing trend means an increase in the amount of yellowness over time (Shafiee *et al*., 2014). The increase in the amount of yellowness can also be explained by the reactions leading to browning. Edible casings can affect the appearance of fruiting bodies. The increase in the a* index during the storage period up to the 14th day can be caused by aging or the progress of the non-enzymatic browning process in the fruiting body. It may also be due to the increase in respiration rate and stimulation of enzyme activities including browning reactions and other reactions responsible for reducing product

quality (Gao *et al*., 2014). Among the investigated treatments, chitosan + cinnamon essences showed a greater effect in reducing the a* and b* indices on the 7th and 14th days of storage. The reason for the color changes of the fruiting body of the uncoated mushroom was the increase in the pH of the product during the storage time, as well as the contact of oxygen with phenolic substances inside the fruiting body and the decomposition of these compounds in oxidation reactions (Mohammadi *et al*., 2021). The chitosan casing containing cinnamon essence prevents the contact of oxygen with the tissue of the fruiting body of the mushroom on the one hand, and on the other hand, by preventing the increase in pH, it prevents the product from reaching the condition of degradation of these pigments (Pirhayati *et al*., 2017).

Soluble solids

The results of variance analysis of the data showed that the effect of time, treatment, and the double effect of time × treatment were significant at the probability level of 1% on the number of soluble solids in mushrooms (Table 2). The average comparison results of data showed that the double effect of time × treatment was significant at the 5% level of Duncan's test on the soluble solids of the mushroom. So, the highest number of soluble solids was observed on the 7th day of storage in chitosan + angelica essence and chitosan + cinnamon essence treatments with 3.53 and 3.48, respectively, which showed a significant increase compared to the control group. By increasing the storage time from day 7 to day 21, the number of dissolved solids showed a significant decrease. The lowest number of soluble solids was observed in the control treatment on days 21, 14, 7, and 0 with 0.86, 0.98, 1, and 1.04 %, respectively. No statistically significant difference was observed between some treatments at the 5% level of Duncan's test.

Table 2. The results of variance analysis of the effect of storage time and treatment on biochemical properties and color indices in *Agaricus bisporus*

Source of variations	Degree of freedom	Mean square				
		Phenolic acid	b^*	a^*	L^*	Soluble solids
Time	3	56673^{**}	349.8^{**}	494.6^{**}	16719^{**}	7.08^{**}
Treatment	4	17863^{**}	1006^{ns}	326.9^{**}	1010^{**}	3.62^{**}
Time × Treatment	12	3554^{**}	266.1^{ns}	125.9^{**}	302.4^{**}	0.60^{**}
Error	40	76.37	22.60	3.64	23.46	0.0004
CV (%)		3.39	26.01	15.25	14.20	1.12

**: Significance at the statistical level of 1%, ^{ns}: non-significance

The results of this research showed that by increasing the storage time to 21 days, the number of dissolved solids showed a significant decrease. Treatments of chitosan + angelica essence and chitosan + cinnamon essence on the 7th day of storage showed the most soluble solids compared to the control group. Soluble solids are important quality indicators that have a direct relationship with the edible quality of the product at the time of ripening. Changes in soluble solids depend on several factors such as fruit sugar content, acidity, and soluble pectins in fruit (Zalewska *et al*., 2018).

The reduction of soluble solids during storage is related to the metabolic activity and respiration of the fruiting body of the mushroom so as a result of these activities, soluble solids (mostly sugars) are consumed (Peña-Estévez *et al*., 2016). The reduction of the total amount of soluble solids during the storage period due to the continuation of the respiration process and the consumption of these compounds to provide energy for the processes was energy-demanding, and the result was a decrease in the amount of total sugar; Because as a result of oxidation, sugars are converted to pyruvic acid and aerobic conversion of pyruvate to carbon dioxide, water, and energy, their amount decreases (Zhang *et al*., 2019).

Edible coatings reduce the rate of aging by reducing respiration and ethylene production and preventing the increase of soluble solids. The results of this research regarding the reduction of soluble solids during the storage period are consistent with the results of the research of Pena-Estevez *et al*. (2016) on pomegranate. The results of the research of Rokayya *et al*. (2021) about the investigation of the effect of nanofilm coating on the biochemical properties of the mushroom showed an increase in soluble solids at the beginning of the storage period and finally a decrease in its amount, which is consistent with the results of this research. Furthermore, the results of this research are consistent with the results of the research of Gholami *et al*. (2020) on the mushroom and the results of the research of Hosseini and Moradinejad (2018).

CONCLUSION

Due to the perishability and browning of the *Agaricus* mushrooms, their preservation is particularly important. The short storage life of mushrooms is an obstacle to their distribution and sale, and increasing the shelf life of mushrooms and maintaining their quality is very important for consumers. The main processes associated with mushroom degradation include membrane rupture, cap opening, blade elongation, and spore formation. This phenomenon, with the browning of the cap and blades and negative quality properties, limits the shelf life of the mushroom after harvesting. One of the methods of preserving the mushroom is covering it with edible casings and treating it with essential oils. In general, by increasing the storage time to 21 days, many qualitative and biochemical properties of the fruiting body were affected. Casings of chitosan + angelica essence and chitosan + cinnamon essence showed the greatest effect in increasing phenolic acid, and soluble solids and decreasing the L* color index. The results of this research showed that the investigation of other concentrations of cinnamon and angelica essential oils and the evaluation of the effect of essential oils alone on

the qualitative and biochemical characteristics of the mushroom may be able to provide different results that can be suggested to the producers of this industry (Table 3; Table 4).

Table 3. average comparison results of the effect of storage time on the biochemical and qualitative properties of *Agaricus bisporus*

(Day) Time	Weight loss (%)	Phenolic acid (mg/kg)	b^*	a^*	L^*	Soluble solids (%)
0	–	234.6 [c]	11.53 [c]	5.28 [d]	83.80 [a]	1.04 [d]
7	5.00 [a]	325.1 [a]	18.07 [b]	14.45 [b]	13.53 [c]	2.70 [a]
14	4.62 [b]	287.6 [b]	21.36 [ab]	18.96 [a]	15.86 [c]	1.96 [b]
21	4.06 [c]	184.4 [d]	22.14 [a]	11.34 [c]	23.23 [b]	1.69 [c]

In each column, means with different letters are significantly different at the five percent level of Duncan's test.

Table 4. average comparison results of the effects of the examined treatments on the biochemical and qualitative properties of *Agaricus bisporus*

Treatment	Weight loss (%)	Phenolic acid (mg/kg)	b^*	a^*	L^*	Soluble solids (%)
control group	1.94 [e]	201.9 [e]	27.05 [a]	12.76 [c]	46.74 [a]	0.97 [e]
Chitosan	2.72 [d]	267.6 [c]	24.46 [ab]	17.96 [a]	36.45 [b]	2.05 [c]
Chitosan + angelica	7.28 [a]	297.5 [a]	21.63 [b]	15.76 [b]	36.37 [b]	2.33 [a]
Chitosan + cinnamon	6.23 [b]	285.1 [b]	4.56 [d]	4.26 [d]	22.47 [d]	2.22 [b]
Chitosan + angelica + cinnamon	4.64 [c]	237.5 [d]	13.70 [c]	11.80 [c]	28.50 [c]	1.67 [d]

In each column, means with different letters are significantly different at the five percent level of Duncan's test.

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