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Evaluation of glucose in Fermentation of *Catharanthus roseus* L. G. Don. Extract by Lactic Acid Bacteria

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ABSTRACT
In this study, two species of lactic acid bacteria (Lactobacillus acidophilus LA5, Lactobacillus casei-01) were used for fermentation of Catharanthus roseus L. G. Don. Extract with glucose addition. Also, the results revealed that probiotics were capable of using the C. roseus extract rapidly for cell synthesis and lactic acid production when glucose was added. However, L. acidophilus LA5 produced a greater amount of lactic acid than L. casei-01 and reduced the pH of fermented C. roseus extract with glucose addition from an initial value of 5.95 to 3.54 after 9 days of fermentation at 37 ºC. After adding glucose to the C. roseus extract, only glucose and fructose were consumed by the fermented C. roseus extracts by L. acidophilus LA5 and L. casei-01 beacause no change was observed in amount of sucrose and maltose. In addition, the study found that the fermentation had a positive effect on the antioxidant activity of the samples. Also, the antioxidant activity significantly ($p < 0.05$) increased by adding glucose to the fermentation. The lowest IC_{50} and the highest FRAP value were 178.41 ± 0.095 µg/mL and 19580 ± 277 µmol Fe^{+2}/L in the fermented C. roseus extract by L. acidophilus LA5 after adding glucose.
Key words: *Catharanthus roseus, Glucose, Fermentation, Probiotic, Antioxidant activity*

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INTRUDUCTION

Generally, glucose is also known as a proper carbon and energy source for many microorganisms [1-2-3-4-5]. Also, fermentation process is used in food industry that can remove undesirable component and improve or preserve the nutritional properties [6]. Furthermore, lactic acid bacteria are used for fermentation process [7]. Some of lactic acid bacteria are probiotics and applied for food fermentations. These probiotics are *Lactobacillus acidophilus, Bifidobacterium* sp., and *L. casei* that improve the health of consumers and quality of the fermented products [8]. Reduction of blood cholesterol, treatment of diarrhea, anticarcinogenic and antihypertensive properties and biotransformation of isoflavone phytoestrogen to improve hormonal balance in postmenopausal women are health benefits of probiotic organisms [8-9-10-11-12].
Catharanthus roseus L. G. Don is a medicinal plant which is rich in phytochemicals and alkaloids [13]. This plant used traditionally for curing various diseases such as diabetes, breast and lung cancer, uterine cancer, melanomas, and Hodgkin's as well as non-Hodgkin's lymphoma [14]. Also, water extracts of *C. roseus* are utilized for diabetes, bleeding arresting, fever or rheumatism [15]. To date of this research, there is no information about the effect of glucose in fermentation of *C. roseus* extract. As the main objective of the current study, the effect of fermentation process on the carbohydrates changes, organic acids as well as the antioxidant activity of the *C. roseus* extract with glucose addition was investigated.

MATERIALS AND METHODS

After obtaining the *Catharanthus roseus* L. G. DON from a local market, it was necessary to eliminate the dirt and soil particles, so the fresh leaves of *C. roseus* were washed with tap water perfectly. By using an oven, the *C. roseus* samples were dried at 40° C for 5 days. Afterwards, the samples were ground using a high-speed Waring blender 7011HS (Torrington, USA) which were later used for the probiotic fermentation.

Ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), sodium acetate trihydrate, acetic acid (glacial)100%, acetonitrile (HPLC grade), ferric chloride ($FeCl_3.6H_2O$) and ferrous sulfate ($FeSO_4.7H_2O$) were all bought from Merck (Darmstadt, Germany). In addition, Glucose (≥ 99.5%), fructose (≥ 99%),sucrose (≥ 99.5%) , maltose (≥ 95%), lactic acid (≥ 99%), acetic acid (≥ 99.9%), citric acid (≥ 99.5 %) were purchased from Fluka (Buch, Switzerland). Finally, De Man Rogosa Sharpe Medium (MRS) broth , De Man Rogosa Sharpe Medium (MRS) agar and peptone water were obtained from Merck (Darmstadt, Germany). Lactic acid bacteria (*Lactobacillus acidophilus* LA5 and *Lactobacillus casei*-01) were provided by Christian-Hansen (Hørsholm, Denmark). Moreover, all the bacterial cultures were kept in a frozen form at -40̊C in MRS medium (Merck, Germany) which contained 20% glycerol.

In this study, the fermentation process was conducted after modifying the method proposed by Wang *et al*. [16]. For this purpose, a 50 g of powdered sample of the *C. roseus* leaves was extracted with 500 mL of boiling distilled water for a period of 20 min. The residual solid was segregated using the filtration after which the clear filtrate was cooled to room temperature. After repeating the procedure three times, the extracts were mixed and glucose (1%, w/v) was also added to this mixture. The obtained clear *C. roseus* was regulated to 1500 mL with distilled water. In addition, the extract was sterilized by means of vacuum filtration while utilizing a 0.2 µm pore membrane. Subsequently, twelve portions of the *C. roseus* infusion with glucose addition (100 mL each) were laid into sterilized twelve 100-mL SCHOTT DURAN laboratory glass bottles with screw caps (SCHOTT DURAN®, Mainz, Germany). Later, the sterilized SCHOTT DURAN laboratory glass bottles containing the *C. roseus* extract with glucose addition were inoculated aseptically with about 10^6 CFU/mL of each probiotic namely the *L. acidophilus* LA5 and *L. casei*-01. Following this procedure, the noninoculated sample (control) and each probiotic culture-inoculated sample were fermented at 37ºC for 9 days in the anaerobic jars that contained Anaerocult A (Merck, Germany). Then, one of the anaerobic jars was removed and the non-fermented as well as the fermented *C. roseus* extract with glucose addition were centrifuged every 3 days. After transferring the super-natant into a 100-mL volumetric flask, the volume was adjusted using distilled water. The resulting solution was employed for analyzing the pH, the LAB enumeration, quantification of carbohydrates, organic acids and finally for analyzing the antioxidant activity.

One ml of fermented and non-fermented samples were diluted serially (1:10) with 0.1% peptone water. Afterwards, they were pour-plated with MRS agar. The agar plates were incubated for 72 h at 37°C in order to determine the probiotic strains populations. Finally, by using a pH meter (Mettler Toledo, Schwerzenbach, Switzerland), the pH value of each fermented and non-fermented sample (10 mL) was quantified every 3 days.

Carbohydrates were quantified using high pressure liquid chromatography (HPLC) according to the method reported by Gadaga *et al*. [17] and Nguyen *et al.* [18] with some modifications. Primarily, a volume of 1 mL sample was added to 0.2 mL acetonitrile (HPLC grade). They were blended and centrifuged at 6000 × g for 10 min. By using 0.2 µm nylon filters (Sartorius, Goettingen, Germany), the supernatant was later filtered. Moreover, 20 µL of the filtrate was infused into HPLC system (Waters Co., Milford, MA, USA) equipped with Refractive Index (RI) detector (Waters 410), and also a pump (Waters 600 contoller). The carbohydrates were then separated at the room temperature by the means of a SUPELCOSIL™ LC-NH_2, (250 × 4.6 mm × 5 µ) column (Supelco, Bellefonte, PA, USA). The mobile phase which was acetonitrile-deionized water (80:20) was used at a flow rate of 1.0 mL/min. Prior to this, the mobile phase was filtered by means of a 0.2 µm nylon filter (Sartorius, Goettingen, Germany). Moreover, the carbohydrate content was determined by utilizing
external standards.

We modified a method which was primarily used by Marsili *et al*. [19] and later by Narvhus *et al*. [20] and employed it in this research for analyzing the organic acids by high performance liquid chromatography (HPLC). After adding 1 mL of the sample to 0.2 mL 0.5 M H_2SO_4 and 8 mL acetonitrile (HPLC grade), they were blended and centrifuged at 6000 × g for a period of 10 minutes. After that, the supernatant filtered after being passed through 0.2 µm nylon filters. Then, 20 µL samples from the filtrate were injected into the HPLC (Waters Co., Milford, MA, USA) using a UV-VIS detector (Waters 486) at 210 nm. Furthermore, by applying an Aminex® HPX-87H (300 × 7.8 mm, 9 µm) ion exclusion column (Bio-Rad Laboratories, Hercules, CA, USA), the separation was performed. Subsequently, the mobile phase, which was 0.009 N H_2SO_4, was used at a flow rate of 0.6 mL/min. Meanwhile, the column temperature was 34 °C. Finally, the organic acids contents were measured by employing the external standards.

Free Radical Scavenging Activity (DPPH) Assay was employed for determining the antioxidant activity. The DPPH disappearance was monitored in this research spectrophotometrically at 517 nm on an EL×800 absorbance plate reader (Biotek, Tacoma, Washington, USA) after applying some modifications to the described procedure [21]. Subsequently, a dilution series was prepared in a 24 well plate surface area for each sample. It needs to be added that the reaction mixtures in the sample wells had 0.5 ml of the

extract and 1ml of 0.2 mM DPPH which were dissolved in 80% ethanol. After adding DPPH, at the room temperature the plate was incubated for 30 min. The absorbance at 517 nm was recorded. Inhibition of free radical by DPPH in percent (%) was estimated by means of the formula below:

% inhibition of DPPH• = {[Ab − Aa]/Ab} × 100

Ab is the absorption of the blank sample while Aa is the absorption of the extract in this formula. It should be maintained that the IC_{50} value will be calculated from a graph plotting inhibition parentage of DPPH• absorbance against the antioxidant concentration [22]. It needs to be mentioned that positive control ascorbic acid was used in this research.

The capability of decreasing the ferric ions was determined by modifying the method described by Benzie and Strain [23]. After adding an aliquot (200 µ) of an extract (with suitable dilution, if necessary) to 3 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM FeCl3.6H2O solution), the reaction mixture was incubated in a water bath at 37 ˚C after which the rise in absorbance at 593 nm was monitored at the 30^{th} min. Ferrous sulfate was utilized as a standard curve. Ferric reducing power of the sample was declared in µmol Fe^{2+}/L.

A completely randomized design (CRD) was used as the statistical design. Furthermore, one-way ANOVA was applied for the data analysis. To emphasize, it is to be declared that all the experiments were done in triplicate whose results were stated as mean ± S.D. (standard deviation). Using MINITAB statistical software release 14 (MINITAB Inc., state college, PA, USA), the data analysis was conducted in this study.

RESULTS AND DISCUSSION

Viable counts and pH changes

During the fermentation of the *C. roseus* extract with glucose addition, the viable counts and pH were monitored in this study [Table 1]. Although the inoculums of the probiotic strains namely the *L. acidophilus* LA5 and *L. casei*-01 were primarily 6.0 log CFU/mL, they rose to more than 8.0 log CFU/mL after 9 days of the fermentation. In this research, the fermentation of the *C. roseus* extract with glucose addition samples had the highest population by *L. acidophilus* LA5. Also, the probiotic strains had an acceptable growth in the *C. roseus* extract with glucose addition. This subsequently yielded a fine substrate for the probiotic growth. Furthermore, there was a significant decrease in the pH values of the fermented *C. roseus* extract after adding glucose samples at the end of the fermentation by *L. acidophilus* LA5 and *L. casei*-01. Organic acids production during the probiotic fermentation process might be responsible for the obtained results. Such results indicate that the pH decrease correspond with the probiotics growth in *C. roseus* extract after adding glucose.

HPLC analysis of carbohydrates

During the fermentation of the *C. roseus* extract when glucose was added, the carbohydrates changes were measured by using HPLC. The results revealed that *L. acidophilus* LA5 and *L. casei*-01 were capable of metabolized fructose and glucose as displayed in [Table 2]. These microorganisms in the study used fructose as a carbon source after glucose addition. At the end of the fermentation process, the maximum fructose consumption was in the fermented *C. roseus* extract by *L. acidophilus* LA5. Based on a report by Srinivas *et al*. [24], the glucose and fructose yielded a positive impact on the growth of *L. acidophilus*. Based on the results, the glucose fermentation ability of the probiotics was responsible for the difference between the glucose content of the products during the fermentation. Also, the glucose concentration declined significantly ($P < 0.05$) during the fermentation [25]. At the end of the fermentation process, no glucose was detected as it had been used by *L. acidophilus* LA5 and *L. casei*-01. Glucose has been reported to be a simple sugar and a vital carbohydrate. A possible explanation of this finding is related to the fact that glucose is rapidly and simply used by these bacteria. In addition, sucrose and maltose did not used by probiotics because the amount of glucose is high. As a result, these bacteria only consumed fructose and glucose as carbone source. Wang *et al*. [26] also stated that for lactobacilli and bifidobacteria glucose is a very fine carbon and energy source.

HPLC analysis of organic acids

There was no detection of lactic and acetic acids in the *C. roseus* extract with glucose addition [Table 3]. Also, it can be clearly seen that the lactic acid which is normally the end product of bacterial fermentation grew during the fermentation of the *C. roseus* extract when glucose was added. It is to be noticed that lactic acid is known as the main metabolite which is generated by lactic acid bacteria [27]. Idris and Suzana [28] highlight that microbial growth determines the lactic acid production; therefore, any increase in the microbial growth will increase the lactic acid production. In this study after adding glucose to the *C. roseus* extract, the microorganism was able to grow and produce lactic acid with the highest efficiency. During the fermentation process of the *C. roseus* extract, the acid acetic content rose when glucose was added. Furthermore, during the fermentation in this study various amounts of acetic acid were formed which depended on the microorganism, substrate amount as well as the fermentation time. After adding

glucose, probiotics grew better and produced more acetic acid. Also, during the fermentation of the *C. roseus* extract with glucose addition, the level of citric acid lowered significantly ($P < 0.05$) after 9 days by *L. acidophilus* LA5. The citric acid decrease was because of being metabolized by probiotics.

Antioxidant activity

Antioxidant phenolic compounds in *C. roseus* are Chlorogenic acid, 5-*O*-caffeoylquinic acid, quercetin and kaempferol [29-30-31-32]. These compounds are natural antioxidant and important group of bioactive compound [33]. Also, antioxidant activity of phenolic compounds is related to their chemical structure and glycosides conjugated with a sugar moiety are present in these compounds [34]. The free forms generally show a higher activity than the combined forms [35]. LAB can break down phenolic glucosides with b-glucosidase and produce free bioactive phenolics [36-37]. As a result, increase of antioxidant activity due to the changes of phenolic compounds after fermentation [38]. Many methods are used for determining the antioxidant activity. In this study, DPPH and FRAP method were employed.

DPPH assay was utilized to assess the free radical scavenging properties of the fermented and non-fermented *C. roseus* extract with glucose addition. [Figure 1] displays the results of this investigation. It was revealed that the lower the IC_{50} value yielded the higher the potential antioxidant activity [39]. Moreover, the IC_{50} values of the fermented *C. roseus* extract with glucose addition in DPPH scavenging activity were significantly lower than the control sample (unfermented). Furthermore, during the fermentation process the decline in the IC_{50} value was significant ($P < 0.05$) for all the samples. Accordingly, it can be concluded that during the fermentation the antioxidant activity rose. Similarly, when glucose was added the IC_{50} value of the fermented *C. roseus* extract by *L. acidophilus* LA5 was lower than the fermented *C. roseus* extract with glucose addition by *L. casei*-01 during the fermentation process. Also, the rise in the FRAP value of the *C. roseus* extract with glucose addition was significant ($P < 0.05$) for all of the products. At the end of the fermentation process, the FRAP value of the fermented *C. roseus* extract with glucose addition by *L. casei*-01 was lower than *L. acidophilus* LA5. After adding glucose, the fermentation process was done well and the fermentation imposed a positive effect on the antioxidant activity of *C. roseus* extract. Also, the fermentation was done well by *L. acidophilus* LA5 in comparison with *L. casei*-01. As a result, the antioxidant activity of the fermented *C. roseus* extract by *L. acidophilus* LA5 was more than the fermented *C. roseus* extract by *L. casei*-01. Finally, the FRAP value of the fermented *C. roseus* extract with glucose addition was 19580 ± 277 µmol/L by *L. acidophilus* LA5. This value was high FRAP which was also close to very high FRAP according to Katalinić *et al.* [40]. As a finding, the fermentation increased antioxidant activity in *C. roseus* extract with glucose addition. It can claim that fermentation increase free phenolic compounds that have antioxidant properties [6-41-42].

Table 1. Changes in viable counts and pH during the fermentation of the *C. roseus* extract with glucose addition [1]

	Time (Day)	Log_{10} CFU/mL [2]	pH [2]
Control	0	ND [3]	5.96 ± 0.010 [a]
	3	ND [3]	5.96 ± 0.005 [a]
	6	ND [3]	5.97 ± 0.010 [a]
	9	ND [3]	5.97 ± 0.017 [a]
L. acidophilus LA5	0	6.42 ± 0.010 [a]	5.95 ± 0.015 [a]
	3	8.03 ± 0.020 [b]	4.02 ± 0.005 [b]
	6	8.34 ± 0.025 [c]	3.77 ± 0.015 [c]
	9	8.58 ± 0.030 [d]	3.54 ± 0.011 [d]
L. casei-01	0	6.44 ± 0.010 [a]	5.97 ± 0.011 [a]
	3	7.70 ± 0.011 [b]	4.31 ± 0.010 [b]
	6	8.00 ± 0.015 [c]	4.07 ± 0.005 [c]
	9	8.23 ± 0.023 [d]	3.87 ± 0.015 [d]

[1] Note: The results are reported as Mean ± SD (n = 3)

[2] Means with different letters within a column are significantly different at $P < 0.05$.

[3] ND: The number of viable probiotic bacteria is below the detection limit (1 log_{10} CFU/mL).

Table 2: Changes in the contents of carbohydrates (g/L) during the fermentation of the *C. roseus* extract with glucose addition [1]

	Time (Day)	Fructose [2]	Glucose [2]	Sucrose [2]	Maltose [2]
Control	0	0.518 ± 0.003 [a]	10.798 ± 0.089 [a]	1.158 ± 0.042 [a]	0.314 ± 0.004 [a]
	3	0.518 ± 0.005 [a]	10.799 ± 0.080 [a]	1.155 ± 0.033 [a]	0.313 ± 0.005 [a]
	6	0.516 ± 0.002 [a]	10.799 ± 0.073 [a]	1.160 ± 0.046 [a]	0.315 ± 0.002 [a]
	9	0.517 ± 0.001 [a]	10.800 ± 0.066 [a]	1.157 ± 0.026 [a]	0.312 ± 0.003 [a]
L.acidophilus LA5	0	0.517 ± 0.003 [a]	10.795 ± 0.099 [a]	1.155 ± 0.037 [a]	0.312 ± 0.005 [a]
	3	0.387 ± 0.004 [b]	4.230 ± 0.042 [b]	1.157 ± 0.034 [a]	0.312 ± 0.006 [a]
	6	0.338 ± 0.001 [c]	1.298 ± 0.031 [c]	1.156 ± 0.027 [a]	0.313 ± 0.004 [a]
	9	0.297 ± 0.002 [d]	0.000 ± 0.000 [d]	1.159 ± 0.033 [a]	0.314 ± 0.002 [a]
L. casei-01	0	0.517 ± 0.002 [a]	10.799 ± 0.087 [a]	1.157 ± 0.036 [a]	0.313 ± 0.005 [a]
	3	0.453 ± 0.006 [b]	6.276 ± 0.051 [b]	1.159 ± 0.031 [a]	0.311 ± 0.004 [a]
	6	0.413 ± 0.003 [c]	3.773± 0.017 [c]	1.158 ± 0.026 [a]	0.315 ± 0.003 [a]
	9	0.374 ± 0.004 [d]	0.000 ± 0.000 [d]	1.156 ± 0.035 [a]	0.313 ± 0.004 [a]

[1] Note: The results are reported as Mean ± SD (n = 3)
[2] Means with different letters within a column are significantly different at $P < 0.05$.

Table 3: Changes in contents of organic acids (mg/L) during the fermentation of the *C. roseus* extract with glucose addition [1]

	Time (Day)	Lactic acid	Acetic acid	Citric acid
Control	0	0 ± 0 [a]	0.00 ± 0.00 [a]	1898 ± 12.7 [a]
	3	0 ± 0 [a]	0.00 ± 0.00 [a]	1897 ± 17.2 [a]
	6	0 ± 0 [a]	0.00 ± 0.00 [a]	1898 ± 15.9 [a]
	9	0 ± 0 [a]	0.00 ± 0.00 [a]	1898 ± 19.6 [a]
L.acidophilus LA5	0	0 ± 0 [a]	0.00 ± 0.00 [a]	1898 ± 13.6 [a]
	3	5265 ± 70 [b]	550.65 ± 2.58 [b]	1695 ± 13.1 [b]
	6	7030 ± 37 [c]	692.42 ± 3.06 [c]	1588 ± 10.4 [c]
	9	7931 ± 56 [d]	822.32 ± 5.34 [d]	1493 ± 13.7 [d]
L. casei-01	0	0 ± 0 [a]	0.00 ± 0.00 [a]	1897 ± 14.7 [a]
	3	4272 ± 66 [b]	431.62 ± 5.05 [b]	1754 ± 16.5 [b]
	6	5242 ± 72 [c]	540.98 ± 3.15 [c]	1653 ± 17 [c]
	9	6128 ± 55 [d]	647.61 ± 4.86 [d]	1559 ± 9.7 [d]

[1] Note: The results are reported as Mean ± SD (n = 3)
[2] Means with different letters within a column are significantly different at $P < 0.05$.

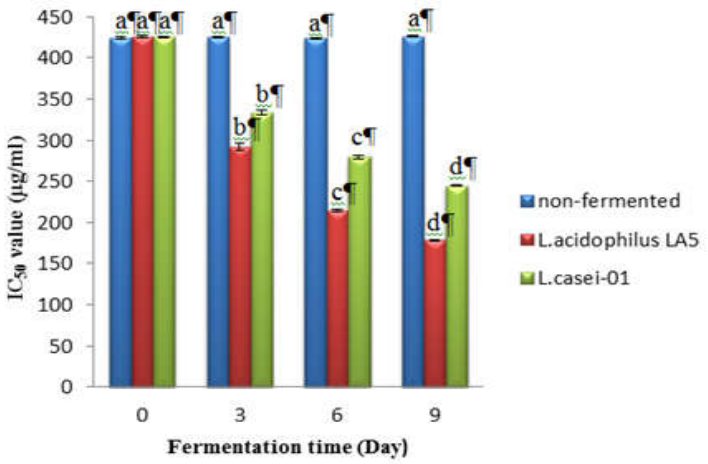

Fig-1. Changes of DPPH radical scavenging in the *C.roseus* extract with glucose addition during the fermentation. Vertical lines show the SD from the mean. a,b,c,d are significant in 95% confidence level.

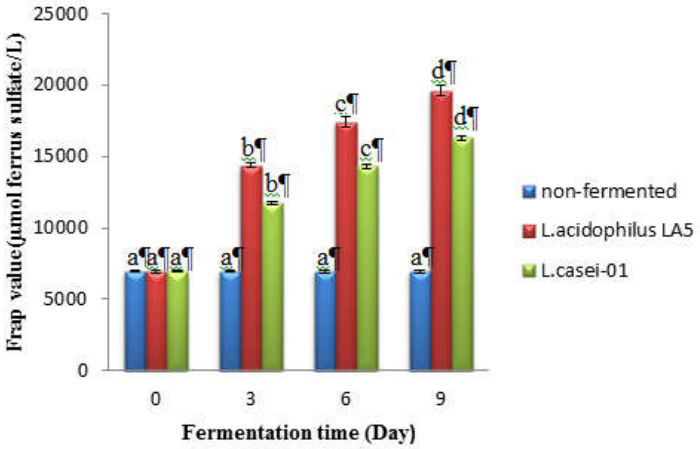

Fig-2. Changes of FRAP value in *C.roseus* extract with glucose addition during the fermentation. Vertical lines show the SD from the mean. a,b,c,d are significant in 95% confidence level.

CONCLUSION

In this study, *L. acidophilus* LA5 and *L. casei*-01 were used as probiotics in fermentation of *C. roseus* extract with glucose addition. They were all able to grow in sterilized *C. roseus* extract with glucose addition and produed lactic acid and acetic acid. This study proved that fermentation of *C. roseus* extract with glucose addition by *L. acidophilus* LA5 was better done as compared with the one fermented by *L. casei*-01. Moreover, the antioxidant activity increased during the fermentation in this study. It can be concluded that the fermentation had a positive effect on the antioxidant activity. Using the results of the present research, fermented *C. roseus* extract with glucose addition can be utilized for isolating high-value bio-actives that might be considered for the development of new drugs and functional foods for pharmaceutical and nutraceutical uses.

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