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THE BIOLOGICAL EFFECT OF MELANIN PIGMENT EXTRACTED FROM *ASPERGILLUS NIGER* IN COLORING LABORATORY BISCUIT

Tiba Basim Mohammed & Alyaa S. Al-Hafud[a]

[a] Department of Home Economics / College of Education for Women / University of Baghdad / Iraq

Abstract

The study was conducted in the Ministry of Science and Technology / Department of Environment and Water laboratories, which dealt with the study of extracting melanin pigment from *Aspergillus niger* fungus. It was isolated from thirteen samples of different foodstuffs. These fungal isolates were diagnosed. Several general of fungi resulting from the food culture process were detected, including *Penicillium spp*. *Aspergillus niger, Emercilla nidalans, Fusarium sp*, and others. *Aspergillus niger* showed clear growth by 50% in rice and tea, 33.3% in dried apricots and dried figs, and 40% in raisins and nomi basra. The final diagnosis of *Aspergillus niger* isolates was confirmed using the polymerase chain reaction (PCR) technique. The molecular examination results showed that most of the isolates contained 18s rRNA with 90% of its genotype. The oral administration results to laboratory mice showed significant differences in liver enzymes. The production of (ALT) enzyme increased at (P□0.05) for the two concentrations of 50% and 100%. Then the enzyme decreased at the concentration of 150%, while there were no significant differences in the production of (AST) and (ALP) enzymes. No significant differences existed in the blood's concentration of creatine and urea. The results of the sensory evaluation of the biscuit treated with biomelanin at concentrations of 50% and 100% showed significant differences at (p□0.05) in appearance, softness and color characteristics. There were no significant differences in texture, flavor and flakeness character.

Keywords: Fungus, Melanin, Genetic Diagnosis, *Aspergillus niger*.

1- Introduction:

The production of pigments from microorganisms is beneficial because it is more practical, efficient and effective in the chemical composition of its. This means that microorganisms are a more viable source than plant and animal pigments because they do not have seasonal differences and do not compete for agricultural land. It can be produced easily and with low production costs [1]. pigments are used in the food industry as a coloring matter, and the pigments extract is used in food as a natural antioxidant taken from a local fungal isolate [2]. It is necessary to choose both the strain and the appropriate fermentation process. So is the choice of media or techniques to improve natural pigmentation [3].

Melanin is one of the pigments that vary from yellow to black depending on the type of melanin, as not all microorganisms can produce this substance [4]. The following fungi (*Aspergillus niger, A.oryzae, D.biseptata, M.cicinelloides, A.versicolor, A.glaucus and P.simplicissimum*) have been identified growing on the skins of cows, goats and sheep [5]. Aspergillus fungus can be isolated, purified and identified from various soil sources, seeds, fruits and vegetables. It is done using

traditional methods that depend on the colony's external appearance and the fungus's shape under a light microscope [6], and the polymerase chain reaction (PCR) technique can be used to diagnose isolates from the *Aspergillus* fungus.

These fungal pigments were used because they are easy to obtain and cheap, and there are studies on their use as they are alternatives to the use of harmful and expensive industrial food dyes. **2- Materials and methods:**

2-1 Isolation of fungi:

Fungi were isolated using the serial dilutions method. Prepared by adding 10 g of sample to 90 ml of physiological solution and mixing using a shaker at a constant speed for 15 minutes. A 0.1 ml pipette was then transferred on to a plate containing sterile PDA medium, spread using a glass spreader and incubated at 25-27 °C for 5-7 days. The isolated fungus *Aspergillus niger* was grown on agar medium (PDA) to obtain a pure culture. Glass slides containing 90% lactophenol blue were observed using a microscope with 100X magnification. The isolate *Aspergillus niger* was identified based on the shape of the colonies, its growth, and sporethe s' shape. Three replications of the dilutions were used using taxonomic keys [7].

2-2 Diagnosis of *Aspergillus niger*:

2-2-1 DNA extraction:

DNA was extracted from 10 samples of the fungus isolate *Aspergillus niger*, using the extraction kit from the equipped company (HiPurA HP Fungal DNA Purification Kit) in India to extract DNA from mold and according to the instructions of the equipped company. The fungus isolates were grown in vitro on a PDB medium. They were incubated in a rocking incubator at 30 cycles/min at a temperature of 25-28°C for 5 days. Then, 3 ml of the liquid culture was transferred to a centrifugal tube at a speed of 13000 x g for 1 minute, the filtrate was discarded, and the sediment (biomass) was taken. Then the precipitate was dried using an oven to identify the genus and species of the fungus *Aspergillus niger*. To obtain a DNA template from a fungus sample, one must go through the extraction stage, where some materials are added to get rid of the cytoplasmic and nuclear membranes and get rid of the associated proteins from the DNA to get a pure DNA template.

2-2-2 Gel electrophoresis:

Prepare 1.5% of agarose in 100 ml of TBE. Then, Ethidium bromide dye was added at a concentration of 5 microliters to the buffer solution. After melting, the agarose is cooled using a water bath at a temperature of 60 °C. The agarose is poured into the device's tray with a thickness of 5-7 mm. Then the comb is placed and left until it solidifies. Then the buffer solution was added until it covered the gel with a height of 2 mm. Then the device was set to 80V for about an hour. After electrophoresis, the gel was taken and placed in a UV transilluminator to examine the DNA bundles after electrophoresis.

2-2-3 Polymerase chain reaction (PCR):

Solutions and materials used in the PCR technology prepared by the company (HP ® HiPurA):

1. E.T. buffer solution.

2. Universal primers (ITS1 - ITS4) for profiling the fungal isolates selected for the ITS region.
3. Master Mix

The solutions and materials were kept at a temperature of -20 C until use .The nitrogen bases of the starter *Aspergillus niger* were sequenced using specialized primers of the fungus. (GATAAAACCATTGTTGTCGCGGTCg)

This technique was used to amplify the ITS region in the DNA that ITS1 - ITS4 primer pairs act on by adding the contents of Table (1) [8]. (GGATTTCGACAGCATTTtcCAAcg).

size	chemical
25	PCR master mix solution
10	Reverse primer
2	Nuclease-free Water
13	DNA
25	Total

After completing all the additions, the samples were mixed centrally using a microcentrifuge. Then the samples were transferred to the Thermal cycler PCR device, then the polymerization reaction was carried out by operating the device and programming it according to the following table:

First: The first stage (Initial Denaturation) is transforming the DNA at a temperature of 95 ° C, and the duration is 3 minutes.

Second: the second stage (annealing) of healing, which includes three degrees of heat, and this step is repeated in thirty cycles in three stages:

1. Transforming DNA at a temperature of 95°C and a period of 45 seconds.
2. Binding of primers to a piece of DNA at a temperature of 55°C for 45 seconds.
3. Elongation at a temperature of 72 °C for 2 minutes.

Third: The third stage (Final Extension) The final elongation is 72 ml for 7 minutes.

Then the cooling step was done at a temperature of 4°C. Then, after the end of the program period, the samples were transferred to the agarose gel.

4-2-2 The method of electrophoresis

Electrophoresis was performed using an agarose gel. [9]. The electrophoresis method was used to detect the products of the. PCR. The agarose gel was placed on the screen of the V.U Transilluminator unit to observe the DNA bundles. Then the results were photographed with a digital camera on the UV unit. Transilluminator. **2-3 Extraction of melanin pigment:**

The melanin pigment was extracted from isolates of *Aspergillus niger*, according to the procedure described by [10]. Using solvents to separate the biomass from the liquid by boiling it with 2 M NaOH at a pH of 12.5 for 36 hours. The centrifuge was set at 4,000 rpm and incubated for 15 minutes. HCl was added to the biomass at a concentration of 2M and pH 2.5 and incubated for

two hours at room temperature. Then it was placed in a centrifuge at 4000 rpm for 15 minutes. The precipitate was then purified with M6 HCl for 2 hours at 100°C to remove carbohydrates, proteins, lipids, and lipids re-dissolved in 2M NaOH and centrifuged at 4000 rpm for 15 minutes. Then the supernatant was deposited and dried
using a 60 °C oven. and collect 48 mg of pigment per 1 mg of biomass.

experiment

2-4-1 Preparation of basic nutrition:

Prepare the laboratory biscuit in the amount of 300 grams. The quantity of biscuits was divided into four equal groups and kept in polyethylene bags, of which three groups were treated with different concentrations (50%, 100%, 150%). The remaining group was left without addition as a control treatment. The groups were preserved until use and given as basic food in an amount of 10 gm for each group per day.

2-4-2 Laboratory animals:

Twelve pure-bred female mice, white in color, aged between 2-4 months and weighing 1520 gm, were used for feeding experiments. Then they were placed in cages with dimensions (21 x 19 x 25.5) cm made of Stainless Steel. The animals were divided into four groups, with three mice per group, as follows:

- The first group (A): the control group was fed on laboratory-made biscuits preserved in polyethylene covers without addition.

- The second group (B): fed on laboratory-made biscuits preserved in polyethylene wrappers treated with melanin dye at a concentration of 50%.

- The third group (C): fed on laboratory-made biscuits preserved in polyethylene wrappers treated with melanin dye at a concentration of 100%.
- The fourth group (D): fed on laboratory-made biscuits preserved in polyethylene wrappers treated with melanin dye at a concentration of 150%.

The experiment lasted 30 days, the temperature at the experiment was 30-25, and the lighting period was at least 12 hours/day. The water was free during the experiment period. The food was prepared as recommended by the Animal House of Biotechnology / Al-Nahrain University.

2-4-3 Examination of liver and kidney function:

After feeding with the pigment-added biscuit, blood was drawn directly by the cardiac pricking method using a medical syringe of 10 ml. Then put the blood in dry and sterile selection tubes. Then the serum was separated using a centrifuge at a speed of 3500 revolutions/min for 15 minutes. The serum was placed in Eppendorf tubes and kept by freezing at -18 ° >

2-5- Laboratory biscuit manufacturing (basic recipe) Materials:

White flour 100 g / 22.7 g fat / 2.7 g table salt / 9 .4g baking powder / 73.6 ml milk.

2-5-1 the methods:

The biscuit was prepared in a laboratory (with some adjustments made in the weights of the materials used) according to the following steps:

1. Sift the flour, salt and baking powder together in a mixing bowl. The oven temperature has been regulated at 218°C.
2. Add the fat to the dry ingredients.
3. Then liquid milk was added to the dry ingredients, then the ingredients were mixed well with a fork several times (about 30 times) until the dough was homogeneous.
4. Sprinkle the wooden board with flour and spread the dough with a thickness of 5 cm in a round biscuit mold of 5 cm in diameter.
5. Place the biscuits in an ungreased mold using a spatula knife, leaving a distance of 1-1.5 cm between the pieces of biscuits until the color becomes golden [11] and add 0.25 g for a concentration of 50% and a ratio of 0.125 g for a concentration of 100%.

3- Results and discussion:

3-1 Isolation and identification of fungi:

The results (Table 2) showed the isolation and identification of fungi associated with thirteen samples of different foodstuffs from Baghdad governorate. The results showed that the fungus *Penicillium spp*. The presence of 100% in the bread sample and the fungi *Aspergillus niger*, *Trichoderma viride,* and *Emercilla nidulans,* with the presence rates of 50%, 30%, and 20%, respectively, in the rice and tea samples. The fungi *Cladosporium sp., Fusarium sp*., and *Aspergillus niger* with an incidence of .333%, .333% and 33.3%, respectively, in dried apricot and dried fig samples. As for the pieces of raisins, nomi Basra, cinnamon and bay leaves, the presence of the following fungi *Aspergillus flavus, Aspergillus niger* and *Penicillium spp.* and *Aeurobasidium sp* with an incidence of 40%, 40%, 10% and 10%, respectively, and the fungi *Alternaria alternata* and *sp. Rhizopus* with 60% and 40% presence, respectively, in samples of white onions, red onions and fresh figs, while the walnut sample was infected with *Aspergillus flavus* only with a percentage of 100%. The isolated fungi varied according to the variation of nutrients between (dry, fresh, and processed), as well as the locations of the sample and the storage period, in addition to the storage periods conditions such as temperature, humidity, light, and a wide spectrum of pH [12]. Studies have indicated the possibility of infection of agricultural crops and foodstuffs with multiple fungal infections. [13] stated that improper practices during and after harvesting agricultural crops and transporting them from the field to the market might lead to infection by several fungal genera, namely *Aspergillus niger* and *Alternaria alternate*. As mentioned, [14], [15] said that the possibility of fungal infection of bread may occur as a result of one of the following steps: transporting bread, during cooling and storage, cutting and packaging.

Table (2) Percentages of fungi isolated from different foodstuffs

percentage	Isolated fungus	samples	No.
% 100	*Penicillium spp.*	bread	1
% 50	*Aspergillus niger*		2

% 30	*Trichoderma viride*	Tea, rice	
% 20	*Emercilla nidulans*		
%33.3	*Cladosporium sp.*	Dry peach Dry fig	3
%33.3	*Fusarium sp.*		
%33.3	*Aspergillus niger*		
40%	*Aspergillus flavus*	raisinNomi Barah ، cinnamon،Bay leaf	4
40%	*Aspergillus niger*		
10%	*Penicillium spp.*		
10%	*Aeurobasidium sp*.		
60%	*Alternaria alternata*	White onion ، red onion ،fresh figs	5
40%	*Rhizopus sp.*		
100%	*Aspergillus flavus*	walnut	6

2-3 Molecular diagnosis of Aspergillus niger

The final diagnosis of the fungal isolates of *Aspergillus niger* obtained from the collected samples was confirmed through the molecular diagnosis of 10 isolates of *Aspergillus niger* using the specific primer of the 18S rRNA gene in their genotype to confirm their diagnosis. This gene also assists in analyzing the species level using the polymerase chain reaction (PCR) technique, as most researchers used the 18S rRNA gene to diagnose *Aspergillus* fungus isolated from different foodstuffs [16]. The molecular examination results showed that most of the isolates contained this gene at a rate of 90% in its genotype, as shown in Figure .)1(

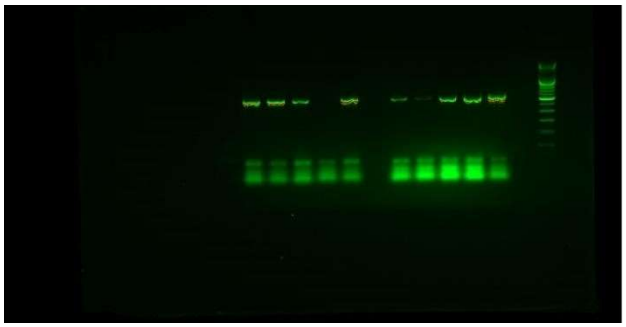

Figure (1) shows the DNA bundles of Aspergillus niger.

[17] stated that the aim of using the ITS regions as targets for evolutionary analysis and study is because they show variation between fungal species but slight variation between strains of the same species. [18] confirmed that the difference in The ITS regions in the molecular study is considered a diagnostic characteristic for each genus and type of fungus, as it contains a sequence of its own, excluding other genera and types of fungi. The results of this study are in line with [19], which mentioned the appearance of the DNA package of *Aspergillus niger* at a molecular weight of 599bp, as well as [20], who found that Aspergillus flavus and *Aspergillus niger* showed DNA bundles at molecular weights bp595 and bp599, respectively.

3-3 pigment extraction:

After completing the molecular diagnosis of the *Aspergillus niger* fungus and then extracting the melanin pigment from the desired fungus, several physical and chemical tests were used to make sure that the extracted pigment is the melanin pigment, including ultraviolet spectroscopy, the absorption of all visible wavelengths, and this is what gives melanin its dark color.

3-4 Results of biochemical experiments conducted on rats

The daily follow-up results during the oral administration of the manufactured biscuit treated with biomelanin at concentrations of 50%, 100% and 150% showed that there were no rat deaths during the 30 days of the experiment, in addition to the absence of any behavior contrary to the nature of these rats. These results are consistent with [21], which reported that no pathological condition or toxic injury appeared as evidence of oral administration of Beauveria bassiana mushroom products to laboratory rats. It also agrees with [22], as he did not show any deaths or pathological injuries through the oral administration of xylindein extracted from *Chlorociboria sp* fungus to zebrafish.

3-5 Effect of oral administration of biscuit treated with biomelanin on liver enzymes of mice

Liver examinations are considered biochemical examinations of liver function by testing the production process of liver enzymes by increasing or decreasing the secretion of liver enzymes in the blood. These enzymes include alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). The results showed, as in Table (3), the effects of oral administration of solid food for each of the biscuits treated with melanin with concentrations of 50%, 100% and 150%, compared to the control treatment on liver enzyme production of rats used during 30 days of the implemented experiment. The results of the statistical analysis showed that there were significant differences in the high productivity of the enzyme (ALT) at ($P \leq 0.05$) for the two concentrations of 50% and 100%, with production values of (23.72 ± 0.83) and (26.50 ± 2.06) IU / liter, respectively, compared to the treatment. The comparison had an enzyme yield of (20.65 ± 1.11) IU/L, and then the enzyme production
decreased at a concentration of 150% to be (24.60 ± 0.61)
IU/L, as shown in Figure (2). While there were no significant differences in the production of (AST) and (ALP) enzymes at

the level (P≤0.05). The productivity of (AST) and (ALP) enzymes at concentrations of 50%, 100%, and 150% was (26.07 ± 0.50), (26.20 ± 2.23), (24.16 ± 2.01), (87.86 ± 2.64), and (84.29 ±). (2.12) and (81.49 ± 1.48) IU/L, respectively, compared to the control treatment, whose production values for both enzymes were (24.47 ± 2.05) and (85.49 ± 5.06), respectively, as shown in Figures (3) and (4). [23].

Table (3) liver enzymes in the blood of mice

Group	Mean ± SE		
	ALT (IU\L)	AST (IU\L)	ALP (IU/L)
Control	20.65 ±1.11 b	24.47 ±2.05	85.49 ±5.06
50	23.72 ±0.83 ab	26.07 ±0.50	87.86 ±2.64
100	26.50 ±2.06 a	26.20 ±2.23	84.29 ±2.12
150	24.60 ±0.61 ab	24.16 ±2.01	81.49 ±1.48
LSD value	4.538 *	6.187 NS	8.935 NS
P-value	0.0413	0.792	0.411
Means that having the different letters in the same column differed significantly. * (P≤0.05), NS: Non-Significant.			

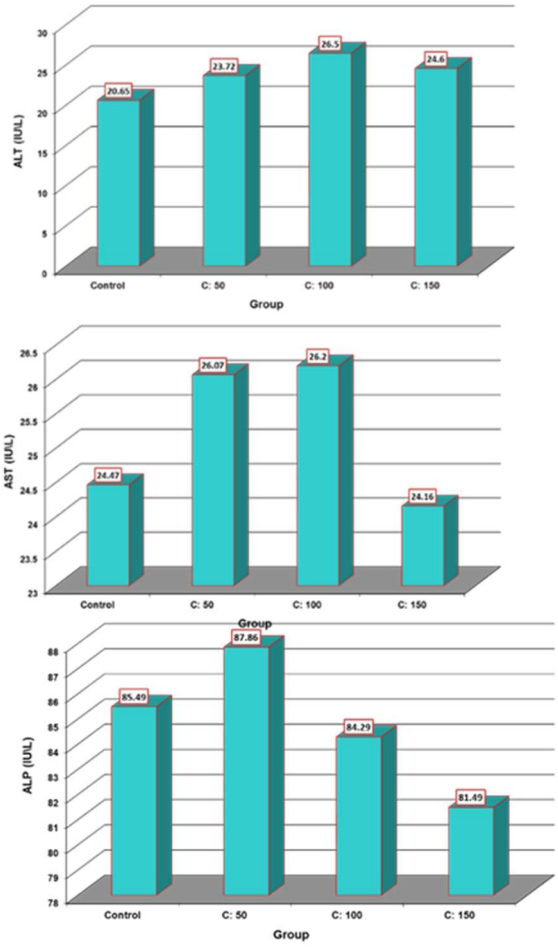

Figure (2) represents a comparison between groups Different in ALT

Figure (3) represents a comparison of groups different in AST.
Figure(4)represents a comparison of the different groups in ALP

[24] also suggested using mushroom melanin as a radioprotective material, especially for cancer patients receiving radiotherapy, as it exhibits anti-radiation activity in vivo. It was stated [25] that natural melanin may also act as an anti-aging drug due to its action in reducing the generation of free radicals, removing excess produced free radicals, and enhancing the activities of antioxidant enzymes. Studies have shown that one of the main causes of aging is the excess of free radicals. It is produced during oxidative metabolism in the human body.

Table (4) compares the different groups in kidney function.

Group	Mean ± SE	
	B. Urea (mg\dl)	Creatinine (mg\dl)
Control	13.37 ±0.94	0.440 ±0.06
50	15.13 ±0.54	0.350 ±0.01
100	15.53 ±1.15	0.373 ±0.02
150	14.57 ±1.43	0.353 ±0.02
LSD value	3.780 NS	0.093 NS
P-value	0.634	0.221
NS: Non-Significant.		

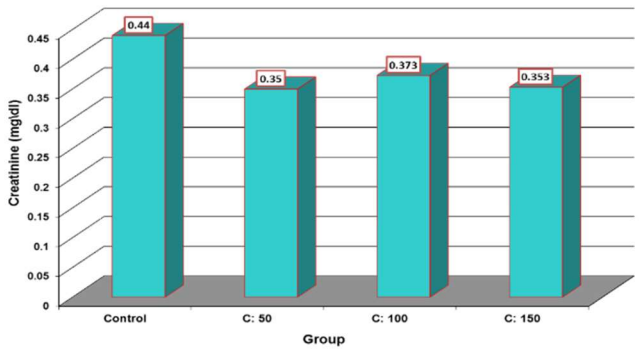

Figure (5) The figure represents a comparison between the different groups of creatinine

Also, the results in the table (4) indicate the concentration of urea in the blood as there were no significant
differences in the concentration of urea in the blood at the attention of 50%, 100% and 150% at the level (P≤0.05), as the values were (15.13 ± 0.54), (15.53 ± 1.15) and (14.57 ± 1.43) mg / dL on respectively, compared to the control treatment with a slightly lower value (13.37 ± 0.94) mg/dL as shown in Figure (6).

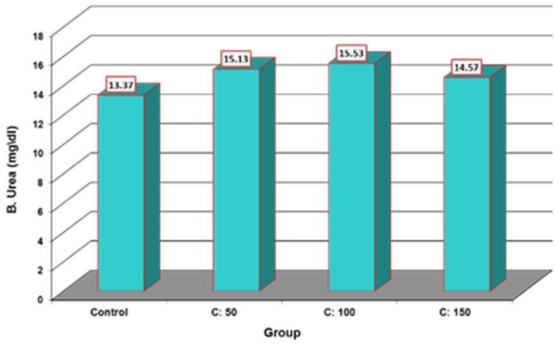

Figure (6) The figure represents a comparison between groups of differences in urea concentration in the blood.

These results came in agreement with [26], who confirmed that biomelanin has a major role in enhancing the activity and work of kidney functions. It also agrees with the researcher [27], who indicated the use of fungal pigments monascin and ankaflavin in a biochemical experiment on laboratory hamsters. The results were to enhance liver function and kidney function.

There were no differences at the level ($P \leq 0.05$), in addition to its ability to reduce cholesterol and triglyceride concentrations, as indicated[28] to the ability of melanin isolate Aspergillus fumigatus to Enhancing kidney function and stimulating the immune system to produce antibodies against some microscopic pathogens.

3-7 Sensory evaluation results

Table (5) shows the sensory evaluation results of the biscuit treated with biomelanin at concentrations of 50% and 100% compared to the melanin-free control treatment. The results showed significant differences at the significant level ($P<0.05$) in the appearance characteristic. The values were (5.6 ± 0.27) and (6.6 ± 0.37) for the 50% and 100% concentrations, respectively. It is less than the value of the comparison

treatment, as it amounted to (6.8 ± 0.52). As for the tissue trait, there were no significant differences between the melanin-treated biscuit and the comparison treatment at a significant level ($P<0.05$). The values for the two concentrations were 50% and 100% (6.2 ± 0.37) and (6.5 ± 0.41), respectively. At the same time, the control treatment value for the same trait was (6.4 ± 0.33). The freshness characteristic showed significant differences between the control treatment and the biscuit treated with melanin at a significant level ($P<0.05$). The value of the control treatment was higher, reaching (6.8 ± 0.54), while the values of melanin scintigraphy for the two

concentrations were 50% and 100% (6.2 ± 0.41) and (5.7 ± 0.35), respectively. They are lower values than the control treatment. As for flavor and flakiness, they did not show any significant differences between the control treatment and the melanin biscuit at a significant level ($P<0.05$). The value of the control treatment was (6.5 ± 0.38) and (6.5 ± 0.35) for each of the two traits, respectively, while the concentration values of 50% and 100% for both traits were (5.6 ± 0.26), (5.5 ± 0.28) and (6.2 ± 0.41) and (6.0 ± 0.34), respectively. As for the color characteristic,

significant differences were shown between the control treatment and the melanin scotch at a concentration of 50% and 100% at a significant level (P<0.05). The values were (6.7 ± 0.51), (5.0 ± 0.22), and (6.7 ± 0.47), respectively.

Table (5) Sensory evaluation of laboratory-made biscuits

Quality / **Total** treatment	**color**	**flakeness**	**flavor**	**freshness**	**tissue**	**shape**
39.7 ±1.45 a	6.7 ±0.51 a	6.5 ±0.35	6.5 ±0.38	6.8 ±0.54 a	6.4 ±0.33	6.8 ±0.52 a
A Cont. 34.8 ±1.22 b	5.0 ±0.22 b	6.2 ±0.41	5.6 ±0.26	6.2 ±0.41	6.2 ±0.37	5.6 ±0.27 b
B ab **50%** 37.0 ±1.06	6.7 ±0.47 a	6.0 ±0.34	5.5 ±0.28	5.7 ±0.35 b	6.5 ±0.41	6.6±0.37 **C** ab
ab **100%**						
3.72 *	1.29 *	0.703 NS	1.008 NS	1.074 *	0.883 NS	1.137 *
value						
LSD						

*Averages with different letters within the same column are significantly different among themselves not significance :NS ,)P<0.05(

The high consumption of biscuits globally made it one of the popular industries, especially for export. Therefore, biscuit manufacturers tried to diversify their products to meet the needs of the market and the consumer. And with the development taking place in the world, it has recently resorted to flavorings and vital dyes, which are environmentally friendly. And for the most important reason is to preserve human health and avoid the dangers and diseases of industrial dyes. The fungal polyketides and carotenoids produced from the fungus
Neurospora crassa have been used as food colorings, which are Generally Recognized As Safe (GRAS) [29] [30] [31] also mentioned the production of red, yellow

and orange fungal pigments from *Monascus s*p., which are safe for health. It was used in the dairy industry as a coloring and flavoring

material, extending the product's half-life. [32] directed the use of melanin pigment produced from the fungus *Aspergillus carbonarius* in the first place. As an inhibitor of free radicals to preserve human health and an environmentally friendly colorant, away from industrial colorants.

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