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Estimating the chemical composition of secondary compounds of Iraqi wild *Agaricus bellaniae* characterized morphologically and genetically

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Abstract:

This study, which is considered the first of its kind in the world and the Arab homeland, was carried out in the laboratory of mushroom production belonging to the Medicinal Plant Unit/ College Of Agricultural Engineering Sciences/ University of Baghdad during the period from July 21, 2016, to December 30, 2018, aiming to isolate and purify the mycelium of the wild isolation in addition to the genetic and morphological identification of the mushroom *Agaricus bellaniae*. The obtained pure isolation was tagged in the American National Center for Biotechnology Information (NCBI) with symbol MF987843.1, thus Iraq would be the second country in the world in which the mushroom is grown following the United States of America. The optimum temperature for the mycelium growth rate was also determined in the laboratory, as they ranged between 50 -60 ◦C. Furthermore, the dried fruit bodies were recognized qualitatively and quantitatively to identify their content of medicinally active compounds. Theyhave shown a high percentage of Linoleic acid (47.77%), total anti-oxidants, and total phenols in addition to the high content of essential chemicals including high protein percentage (44%), mineral elements- selenium in particular (0.369 ppm), and amino acid where glutamic and aspartic acids recorded the highest percentage, reached 4.02% and 2.226% respectively.

Keyword: *Agaricus bellaniae* , phenotypic , molecular structure, active constituents.

Introduction:

The mushroom *Agaricus bellaniae* belongs to the Basidiomycota group, order *Agaricales*, and family *Agaricaceae* which is listed among edible mushrooms. It was classified as edible mushroom that grows during late summer to early fall season within weeds in the form of cuts, arches, or circles distributed in the huge eastern plains of Illinois in the USA [1] .This type of mushroom is characterized by the short, convex, and bell-shaped cap in the early stages and then, at the maturity, it expanded to become flat with inverted convex from the middle to the inside. The cap diameter is 3.5-8 cm containing yellowish-brown gills in contrary to the color of the cap, which is pale brown in color at the edges and dark brown in the middle that grades in color during maturity. The cap edge is soft and bump-free while the gills are free at the stem region. They are short and compact at initially with a white color turns to pink in the center of the cap at the aging ending in brown color at the button stage when the brittle, white ring appears on the stem. The stem height is 3.5 -7cm and its diameter 5-12 cm either uniform or widening at the base. Stem color turns to brown during aging, and sometimes pink bumps appear at the base. The flesh is white unchanging color after cutting. The spore print color is dark brown [2] .

The importance of edible genus *Agaricus* collected from the wild and consumed by humans is due to the high nutritional value, medicinal compound content, taste, and flavor. This mushroom genera included *A. augustus* Fr., *A. campestris* L.: Fr, *A. arvensis schaeff*, and *A subrufescens*[3] however, *A.bisporus* is regarded the commonest species among them for the nutritional value it has as it is characterized by the high content of protein in the fruit bodies that is around 11.01- 29.14 % varying according to the growth culture media [4].Total

carbohydrates content was 51.05% involving the digestible carbohydrates such as mannitol and glucose, usually forming less than 1%, in addition to glycogen forming 5-10% of the dried fruit bodies while the indigestible carbohydrates, including oligosaccharides such as trehalose and non starch as well as the polysaccharides such as lignin, beta-glucan, and mannans, formed the major part of the carbohydrates found within the cultivated mushroom [5] . Mannitol and trehalose are among the saccharides abundantly found in the cultivated mushroom of both types, the white and the brown. The dried fruit bodies of *A.bisporus* (cultivated mushroom) also contain chitin, hemicellulose, mannans, and Beta-glucan which have beneficial characteristics for human health [6]. Rukaibaa [7] mentioned that the fruit bodies of the cultivated mushroom contain k, Fe, Zn, Cu, Na, Se, Mn, Cad, and Co where the amount of phosphor, calcium, magnesium, sodium, iron, and zinc is usually high in the dried fruit bodies . Ekhlas et al [6] found that the polysaccharides content in the dried fruit bodies of the cultivated white mushroom of the brown series enhances the immunity system in a human body and acts as an anti-tumors in-vitro as well as in-vivo., furthermore, the dried bodies of them contain anti- filamentary and anti-oxidants such as phenols and chitosan that prevent the fatty liver disease [8] as well as they contain several anti-bacterial compounds [9]. This mushroom has been recognized in North America through a study prior to this and symbolized at the National Center for Biotechnology Information (NCBI), with the symbols NR_145001.1, KJ877783.1[10], and KJ877782.1 . Therefore, conducted for the first time in Iraq, this research aims to determine the nutritional and medicinal compounds in the mushroom *Agaricus bellaniae* which grows wildly in the middle region of Iraq at the high temperature weather.

Material and methods:

Samples collection: The samples of *Agaricus bellaniae* were obtained from the Salhia region, Baghdad where the temperature was about 50-55 °Con July 21, 2016 (Fig.1). They were propagated and purified on Potato Dextrose Agar (PDA) media [11] in the Mushroom Laboratory of the Medicinal and Aromatic Plants Research Unit at the University of Baghdad, College of Agricultural Engineering Sciences where the isolation purification period lasted virtually for a year.

Figure 1.The wild agricultural mushroom *Agaricus bellanniae*

Wild mushroom diagnosis:

a-Morphological diagnosis

Morphological characterization of mushrooms samples was taken *in situ* Macro fungi characterized using coloured field guide books, photographs, monographs and published work [12] as well as databases. Conventional characterization was based on the features such as photograph, colour Spore print,stalk length, stalk diameter and cap diameter, ecological and host substrate specificity

b-Molecular identification of Wild mushroom

DNA extraction was done using the equipment listed below:

ZR Fungal / Bacterial DNA MiniPrep ™, Catalog No. No. D6005 PreMix was used to duplicate the diagnostic gene ITS4 and the following primers were added (Table 1).

Table 1. Primers were added

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	TCCGTAGGTGAACCTGCGG	60.3	50 %	650 base pair
Reverse	TCCTCCGCTTATTGATATGC	57.8	41 %	

The following program was chosen for gene amplification

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	94°C	3 min	35cycle
2-	Denaturation -2	94°C	45sec	
3-	Annealing	60°C	45sec	
4-	Extension-1	72°C	45sec	
5-	Extension -2	72°C	10 min.	
6-	Cooling	4	∞	

Electrophoresis of PCR products

Preparation of agarose gel:

The gel was prepared at a concentration of 1% by dissolving 0.5 g of acarose in 50 ml of 1 x TBE solution, heated using a microwave oven for 2 minutes, cooled to approximately 55 °C and adding 2 µl of ethidium bromide dye to it.

Preparation of gel mold and sample

Pour the gel at 55-50°C into the electric transfer mould. Place the comb at the end of the gel mould, after the end of the mold is blocked, and leave for half an hour to solidify. Then, remove the comb and add 1× TBE buffer to cover the surface of the gel.

After PCR reaction, the samples results were electrophoresis on agarose gel 1.5% and sent to Macrogene Co. thatgave us nucleotides sequence for each isolate , this sequence enteredthe blast program to find the similarity between sequences of the isolates, while all isolates are recorded in NCBI.

investigating the best extent of mycelium growth of the mushroom *Agaricus bellaniae* from the mother culture in vitro.

The mycelium growth in PDA was studied within various temperature extents including the following seven treatments(25,30,35,40,45,50,55,60) °C

The parameters included are:

- Number of days for completing the colony diameter.
- Number of days required for mycelium growth commence.

The experiment involved culturing 5 dishes where the results were analyzed with Genstat software according to the Completely Randomized Design (CRD).

investigating the chemical composition and the content of the medicinal secondary compounds of the mushroom.

The remained part of the fruit body samples were dried and ground with a special grinder. Next, the samples were placed in tightly closed containers prepared for this purpose, and after that, they were sent to the Arab Republic of Egypt, Agricultural Research Center, Regional Food and Feed Center, to analyze the chemical elements and compositions and probe the percentage of medicinal compounds with GC-mass device where the following estimates have been involved.

1. The medicinal compounds content in the fruit bodies estimated with the GC-mass device.
2. The total antioxidants content, total phenols content and total flavonoids content in the dried fruit bodies calculated according to the procedure described by Albaldawi et al [13].
3. The content of the chemicals in the dried fruit bodies calculated according to the procedure described by Cho et al [14].

Results and discussion:

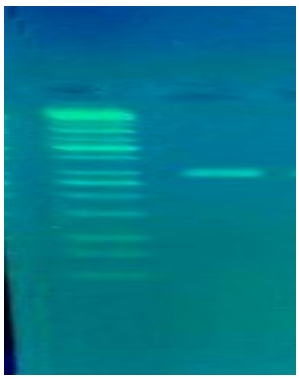
The sample of *Agaricus bellaniae* was obtained from Salhia region, Baghdad where the temperature was about 50-55 °Con July 21, 2016. They were propagated and purified on Potato Dextrose Agar (PDA) media [10] in the Mushroom Laboratory of the Medicinal and Aromatic Plants Research Unit at the University of Baghdad, College of Agricultural Engineering Sciences where the isolation purification period lasted virtually for a year. The morphological traits of the wild mushroom bodies were identified as illustrated in Table 2.

Table 2. Morphological identification the fresh bodies of the wild mushroom *Agaricus bellaniae*

Cap shape	**Short, convex, and bell-shaped in the early stages and then, at the maturity, it expanded to become flat with inverted convex from the middle to the inside.**
Cap diameter	About 4-8.7 cm
Cap color	Outer surface is dark brown in the middle, graduates to pale at the cap edges, while the inner part is characterized by its white flesh
Gills	Free at the stem region with white color at the edges and pink in the center of the cap at the commencing and then turn to dark brown at aging.
Ring	Brittle, white in color appears on the stem beyond the button stage completion
Stem	Heighted about 3.1-7.8 cm with 4.6-11.8 mm in diameter, widening at the base, browning in color during aging, and has pink bumps near the base.
Spore print	Dark brown

The fungi molecular diagnosis was made by 18S with ITS1 and ITS4 primers usage . After PCR reaction samples results were electrophoresis on agarose gel 1.5% and sent to Macrogene Co. While Macrogene Co repeated a nucleotides sequence for each isolate, this sequence enteredthe blast program to find the similarity between sequences of the isolates.

This diagnosis relied on the gene ITS4 gene. Firstly, the fungus' genetic material was extracted and checked for purity, and then it was amplified by PCR using the gene mentioned above. One package appeared, representing the whole genome extracted from the fungus. ITS4 gene amplification was done using the mentioned primers and the prepared program for this purpose. Fig. 2, shows gel electrophoresis of DNA extracted from fungal isolates; a single bind appeared at the site 600 - 700 bases and in two replicates [15]



M F1

Figure 2. Electrophoresis of the amplification product of the ITS4 gene showing the location of the segment between the molecular weight 600-700 nitrogenous bases

This bind was sent to the Korean company (Macrogene) to show the sequence of nitrogenous bases. Upon analyzing these results in the BLAST program, it was found that the isolate under study was related to *Agaricus bellanniae*, and the isolate matched with an isolate that had been registered in the genebank with the code NR145001.1. The strain under-study was recorded in the genebank with the searchers' names as a new isolate of *Agaricus bellanniae* and was given the international code MF987843. It is to be noted that this strain of fungus is being diagnosed for the first time in Iraq and the Middle East.

Table 3, shows that the control needed time more than other “treatments” and the highest degree of growth was at the highest temperature, 50 °C and 55 °C recording the superiority in the number of days required for completing the mycelia growth that was 3.0 and 3.3 days respectively, while the control treatment needed more days reached 12.3 days to complete mycelia growth.The treatments of the incubation temperatures 40, 45, 50, 55, and 60 °C show a remarkable superiority compared to the 35 °C and the control treatments in the number of days required for commencing the mycelium growth on the culture media achieving 2.3, 2.0, 1.7, 1.3, 1.3, 4.3, and 3.7 days respectively.

Determining the best temperature degree for mushroom genus *Agaricus* growth as well as the other edible genera is considered essential and necessary for affecting the production process economically as a result of its relation to the mycelium growth rapidity at the spawn production stage and to its growth rapidity on the compost cultivation media during producing the fruit bodies of the genus *Agaricus*[16].

Table 3. Effect of incubation temperature on the number of days required for completing the colony diameter (days) and the number of days (days) required for commencing mycelium growth of the mushroom *Agaricus bellaniae* in the laboratory on a culture media

Mycelium growth temperature	Number of days required forcompleting the colony diameter (days)	Number of daysrequired for commencing the mycelia appearance on the culture media (days)
Treatment of 25°C (control)	12	4
Treatment of 30°C	9	4
Treatment of 35°C	8	3
Treatment of 40°C	6	2
Treatment of 45°C	5	2
Treatment of 50°C	3	1
Treatment of 55°C	3	1
Treatment of 60°C	4	2
L.S.D(0.05)	2	1

Table 4, demonstrates that Linoleic acid content in the dried fruit bodies was as the highest percentage that recorded 47.77 %. Linoleic acid is defined as a polyunsaturated fatty acid used in the biosynthesis of arachidonic acid (AA) as well as some other substances such as Prostaglandin, Leukotrienes (LTA, LTB, and LTC), and Thromboxane (TXA). All these linoleic acid products have a biological activity associated with human physiology and pathology. Linoleic acid is an essential fatty acid for humans, which must be obtained through diet forgood health. During an experiment on mice, lacking linoeate in the feed resulted in slight expansion in the skin, hair loss ,and poor wound healing , furthermore, it is considered an anti-cancer [16]. The percentage of sinaply alcohol was 0.99 7%. It is an organic compound related to cinnamic acid, one of the mono lignin that is considered the initiator for the biosynthesis of different stilbenoids and coumarins, while the percentage of Melezitose was 0.51 %. This compound is molecularly decomposed into glucose and turanse, sucrose isomer, [17]. The percentage of the flavone complex Scutellarein was19.3% which importance lies as an anti-ischemic and anti- heart disease in humans [18]. The compound 4',6-Dimethoxyisoflavone-7-O-B-D was at the percentage of 1.15% which importance lies as a secondary compound regulates the fat metabolism in the liver and reduces the insulin allergy [19]. The percentage of Zearalenone was 0.77% that is one of the mycotoxins acting as an anti-oxidant leading to the programmed death of cancer cells [20]. Glycitin recorded 0.67 %, which is a glycosylisoflavone compound characterized by its activity inhibiting the cancer cell growth [21]. The percentage of Cholesta-4, 6-dien-3-one reached 0.87%. It is an important compound, as this was proven through laboratory experiments in mice, it reduces cholesterol in the body [22]. The Ethyl linoleate percentage reached 11.42%. It acts as an anti-fungal and anti-oxidant compound [23]. The percentage of Quercetin 3',4'-7-trimethyl ether was 0.92%. It is a flavone belonging to the polyphenols helping to prevent neurodegenerative diseases and inhibit six types of cancer. The percentage of γ-Sitrosterol was 0.89%. It is characterized by the co-effect on the human immune system and the protection against many diseases [24]. The percentage of oleic acid was 1.1 %. This fatty acid is considered as anti-inflammatory compound. Luteolin6, 8-C-diglucoside recorded the percentage of 1.1 %. It is one of the monounsaturated fatty acids known as omega 9 that is considered an anti-oxidant helping to decrease hypertension in addition to enhancing the cell membrane construction and brain development [25]. The percentage of vitamin E was 1.67%. This vitamin is known as anti-inflammatory and anti-oxidant which is related to many diseases and cancer types such as prostate and skin cancer, moreover the skin diseases such as eczema[26].

Table 4. Percentage of the medicinal compound contents in the dried fruit bodies of *Agaricus bellaniae* estimated with the GC-mass device

No	Medicinal compound	Retention time (min)	Percentage (%)
1	Sinaply alcohol	5.3	5.3
2	Melezitose	11.3	11.3
3	Arachidic acid	12.57	12.57
4	Phytol acetate	12.93	12.93
5	Cis-13,16-Docasadienoic acid	13.1	13.1
6	5,7,3',4',5'Pentahydroxyflavone	13.3	13.3
7	Santalcamphor	13.65	13.65
8	Scutellarein	13.77	13.77
9	4',6-Dimethoxyisoflavone-7-O-B-D glucopyranoside	13.88	13.88
10	Zearalenone	14.27	14.27
11	Glycitin	14.35	14.35
12	Cholesta-4,6-dien-3-one	14.4	14.4
13	Cis-Vaccenic acid	14.5	14.5
14	Linoleic acid	14.78	14.78
15	Ethyl linoleate	14.9	14.9
16	Quercetin 3',4'-7-trimethyl ether	16.38	16.38
17	γ-Sitrosterol	16.9	16.9
18	Oleic acid	17.9	17.9
19	Luteolin 6,8-C-diglucoside	18.5	18.5
20	14-β-H-Pregna	19.46	19.46
21	Vitamin E	20.07	20.07
22	Phytanic acid	20.7	20.7
23	Trans-Geranylgeraniol	21.8	21.8
24	Curcumol	21.98	21.98
25	Stigmasterol	22.17	22.17
26	Heptacosane	23	23

The phytanic acid showed a percentage of 1.16%. It is one of the saturated fatty acids reducing prostate cancer occurrence [27].The percentage of Trans Geranyl geraniol was 0.82%. This compound prevents the poisonous effect of cholesterol on cells without reducing the beneficial effect [28]. Curcumol which reached 1.02% reduces the incidence of breast cancer [29]. The percentage of Stigmasterol was 1.02%. It decreases cholesterol as a result of reducing its absorption since it is considered an anti-cancer of colon, ovary, prostate, and breast [30].

The medicinal active materials of which percentages were measured with the GC mass and listed in Table 3, show the importance of active materials in this mushroom compared to those found in *Agricus bisporus* that were recognized by many other investigative studies [31].

Table 5, ilustrates the content of total antioxidants, total phenols, and total flavonoids, in the fruit bodies of *Agaricus bellaniae*, to which the medicinal importance of the mushroom is due unlike what is found in the common species, *A. bisporus*[22]

Table 5. Contents of the total anti-oxidants, total flavonoids, and total phenols in the dried fruit bodies of *Agaricus bellaniae*

No	Medicinal compound	Dry matter concentration of the dried fruit bodies (mg/100g)
1	Total antioxidant	10006.4
2	Total Phenols	1120.7
3	Total flavonoids	92.4

Results in Table 6, illustrate the nutrient value of the mushroom *Agaricus bellaniae* showing the superiority the protein percentage in the fruit bodies compared to its content in white mushroom, *A.bisporus* Results in Table 5, ilustrate the nutrient value of the mushroom *Agaricus bellaniae* showing the superiority the protein percentage in the fruit bodies compared to its content in white mushroom, *A.bisporus* explaining the decrease in the total carbohydrate percentage unlike what was mentioned in researches referring the total carbohydrates are high in the grown mushroom[32]. Though the oil percentage in the studied mushroom was high, most of the fatty acids were beneficial in the sense of health for humans that was confirmed by the results illustrated in Table 4

Table 6. Percentage of the nutritional contents in the dried fruit bodies of *Agaricus bellaniae*

No	Nutrient ingredient	Percentage (%)
1	Proteins	44%
2	Fats	4.17%
3	Raw fibers	13.33%
4	Total carbohydrates	37%
5	Ash	1.10%

Table 7, shows the content of minerals elements in *Agaricus bellaniae* which play an important role in the human health especially selenium, which regarded one of the essential anti-cancer elements, recording a remarkable increase reached 0.3691 ppm compared to *A.bisporus* that according to previous studies its dried fruit bodies contained 1.34 mg/kg [33].

Table 7. Content of some mineral element in the dried fruit bodies of *Agaricus bellaniae*

No	Element	Concentration (ppm)
1	**Sodium**	380.02
2	**Selenium**	0.3691
3	**Copper**	42.74
4	**Phosphorous**	0.59
5	**Calcium**	0.134
6	**Potassium**	46862.42
7	**Iron**	304.9
8	**magnesium**	342.7
9	**Zinc**	267.10

The results in Table 8, demonstrate the amino acid contents in the dried fruit bodies in the wild mushroom where the highest percentage was recorded by Glutamic acid 4.02% followed by Aspartic acid 2.26%. The data in the same table show that the powder of the dried fruit bodies contained 16 amino acids beneficial for human nutrition. Glutamic acid is necessary as it enhances the immunity system of the human body and maintains the health of newborns, adults, and pregnant women as well as it maintains the integrity of the membrane surrounding the digestive system [34,35]. Aspartic acid is distinguished by being involved in synthesizing and secreting hormone testosterone, which is responsible for fertility in men and important for athletes, as it participates in building muscles and increasing their strength [36,37].

Table 8. Content of amino acids in the dried fruit bodies of *Agaricus bellaniae*

No	Amino acid	Percentage (%)	No	Amino acid	Percentage (%)
1	Aspartic	2.26	10	Tyrosine	0.95
2	Therionine	1.09	11	Phenylalanine	1.07
3	Serine	1.19	12	Histidine	0.63
4	Glutamic	4.02	13	Lysine	1.98
5	Glycine	1.16	14	Argnine	1.41
6	Alanine	1.04	15	Proline	0.81
7	Valine	1.44	16	Cystine	0.34
8	Isoleucine	1.15	17	Methioineine	0.46
9	Leucine	-			

Conclusion:

According to the high medicinal and nutritional value of *Agaricus bellaniae* represented by this study, the results show that the dried fruit bodies are rich in secondary compounds such as 47.77% Linoleic acid and 0.369 ppm of selenium which are considered as anti-cancer compounds in addition to sodium and potassium which play an essential role in decreasing the hypertension of human. The dried fruit bodies of this mushroom are also distinguished by the high percentage of glutamic acid 4.02% and aspartic acid 2.26% which are important for human health. Therefore, according to the findings obtained, we recommend producing the mushroom on a commercial scale, taking into account introducing the treatments that increase the valuable medicinal and nutritional materials of the medicinal importance for the human body.

Authors' Declaration:

- Conflicts of Interest: None.

- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad, Iraq.

Authors'contributions statement:

The project has been done in College of Agricultural Engineering Sciences,University of Baghdad. Z M.A-Q and RA.C wrote the manuscript .ZM.A-Q performed the statistical analysis . Z M.Al-Q and R A.C discussed the results and contributed the final manuscript.S k.M.A-T participated in the isolation coding of the fungi in this research in NCBI American organization .

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تقدير التركيب الكيميائي للمركبات الثانوية للفطر البري العراقي *Agaricus bellaniae* وتشخيصه مورفولوجيا وجزئيا

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الخلاصة:
هدفت هذه الدراسة التي تعد الأولى من نوعها في العالم والوطن العربي التي أجريت في مختبر أنتاج الفطر التابع لوحدة بحوث النباتات الطبية/ كليةعلوم الهندسة الزراعية– جامعة بغداد للفترة من 21 تموز 2016 ولغاية 30 كانون الأول 2018 إلى عزل وتنقية الغزل الفطري للعزلة البرية ، ثم إجراء التشخيص المظهري والجيني لها ،إذ اظهر ان العزلة البرية تعود للفطر *Agaricus bellaniae* بعدها تم ترميز العزلة النقية التي تم الحصول عليها في منظمة NCBI الأمريكية بالرمز MF987843.1وبذا يكون العراق ثاني دولة في العالم ينمو فيها الفطر بعد الولايات المتحدة الامريكية ،كما تم تحديد درجات الحرارة المثلى لسرعة نمو الغزل الفطري مختبريا ،إذ تراوحت مابين50 -60م ،كما تم اجراء التشخيص النوعي والكمي لمحتوى الاجسام الثمرية المجففة للفطرالبري لمعرفة محتواه من المركبات الفعالة طبيا ،إذ اظهرت ارتفاع نسبة كل من Linoleic acid47.77 % و مضادات الاكسدةالكليةوالفينولاتالكلية والفلافونويدات الكلية ، فضلا عن محتوى الاجسام الثمرية من المركبات الكيمائية الاساسية وخصوصا ارتفاع نسبة البروتين 44% والعناصر المعدنية خصوصا عنصر السلينيوم0.3691ppm والاحماض الامينية والتي سجل كل من حامضي الكلوتاميكوالاسبارتيك نسبة مرتفعة بلغت 4.02 % و2.26 % على التوالي.

الكلمات المفتاحية: الفطر ، الشكل الظاهري ، التركيب الجزيئي ، المكونات الفعالة.