Studies on the sclerotia of *Aspergillus sclerotioniger* and the alkaloid extracted

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ABSTRACT

In the present study the sclerotia of different Aspergilli (*Aspergillus sclerotioniger, A. sclerotiorum, A. candidus, A. flavus, A. piperis, A. ochraceus, A. robustus, A. sepultus, A. petrakii, A. melleus, A. parasiticus and A. sclerotiicarbonarius*) were examined for the presence of alkaloids in their formed sclerotia separately where, *A. sclerotioniger* exhibited the best result. Accordingly, the culture conditions (temperature, pH, carbon source, nitrogen source and carbon/nitrogen (C/N) ratio were optimized for the formation of sclerotia by *A. sclerotioniger*. The best weight of sclerotia was achieved at temperature 30°C, PH 6, maltose as carbon source, KNO_3 as nitrogen source and (C/N) ratio of 43.33.

The total alkaloids content of *A. sclerotioniger* sclerotia was detected using Van Urk's reagent and recording the optical density on spectrophotometer at 580 nm, then separated on thin layer chromatography (TLC). The separated alkaloid was defined using the percentage of carbon, hydrogen and nitrogen (CHN) percentage, as well as ultraviolet (UV) spectrum, nuclear magnetic resonance (H1NMR) analysis, gas spectroscopy mass spectral analysis (GC-MS mass) and infrared (IR) analysis. The alkaloid was found to be a member of indole alkaloids and was defined as chloro derivative of aflavinine.

Key Words: *Aspergillus sclerotioniger; Sclerotia; Alkaloids; Culture conditions*

INTRODUCTION

Sclerotia are resting bodies produced by several fungi as a mechanism for their long-term survival and propagation of the species [1,2]. The sclerotia are morphologically variable but they are nutrient-rich of multi-hyphal structures which enable them to remain dormant or quiescent when their environment is adverse; and then when conditions improve, germinate to reproduce the fungus [3]. Several species of black Aspergilli are able to produce sclerotia including *A. carbonarius, A. ellipticus, A. aculeatus, A. costaricaensis, A. piperis, A. sclerotioniger, A. aculeatinus* and *A. sclerotiicarbonarius*. Among them, *A. sclerotioniger* give positive result with Ehrlish reagent at the sclerotia region which means that the sclerotia contain alkaloids [4]. Ergot alkaloids are a complex family of indole derivatives with diverse biological activities [5] and produced mainly by fungi of the genera *Claviceps, Penicillium and Aspergillus* [5,6]. These ergot alkaloids and related indole metabolites were considered to protect the sclerotia from predation especially those of the genus *Claviceps* [7]. These important indole metabolites are also associated with the sclerotia of Aspergilli such as, *A. flavus*, but not the vegetative mycelium or conidia [8]. In the meantime, three new diketopiperazine metabolites (N-methylepiamauromine, epiamauromine and cycloechinulin) were distinguished from the sclerotia of *A. ochraceus* [9]. Also, variecolactol was isolated from the organic extracts of the sclerotia of *A. auricomus* [10]. On the other hand, three new metabolites with the aflavinine ring, were isolated from the sclerotia of *A. tubingensis* (NRRL 4700), and their structures were assigned primarily through nuclear magnetic resonance (NMR) studies [11]. The crude extracts of the sclerotia of *Aspergillus niger* showed effective insecticidal activity against the corn borer, *Ostrinia furnacalis* [12].

A. sclerotioniger is a member of *Aspergillus niger* group which was discovered in 2004 [13]. This fungus produces distinct globose to subglobose and red brown sclerotia. For collecting more knowledge about this species few researches were conducted concerning its physiological characters. Accordingly, this work was designed to study the sclerotia formation of different Aspergilli (*Aspergillus sclerotioniger, A. sclerotiorum, A. candidus, A. flavus, A. piperis, A. ochraceus, A. robustus, A. sepultus, A. petrakii, A. melleus, A. parasiticus and A. sclerotiicarbonarius*) concerning the alkaloid extracted. On the other hand, the study included the mycelial growth and formation of sclerotia of *A. sclerotioniger* with special attention to the alkaloid compounds in these sclerotia as well as factors affecting their production.

MATERIALS AND METHODS

The tested fungi

For the present study some sclerotia forming Aspergilli were chosen. *Aspergillus sclerotioniger, A. sclerotiorum, A. candidus, A. flavus, A. piperis, A. ochraceus, A. robustus, A. sepultus, A. petrakii, A. melleus, A. parasiticus and A. sclerotiicarbonarius* were purchased from the mycological center, Assiut University, Egypt and maintained on Czapek's agar medium (CZA).

Chemicals and standard alkaloids

P-dimethyl-amino-benzaldehyde was used in preparation of color reagents. It was purchased from El Gomhoria Company, Cairo, Egypt. Pure samples of α-ergocryptine and ergocornine were purchased from Sigma-Aldrich Company and used as standard alkaloids.

Media and reagents

CZA medium was used in the present study. The citrate-phosphate and boric acid-borax buffers were prepared according to references [14] and [15] for adjusting the pH values of CZA medium. On the other hand, Ehrlich's and Van Urk's reagents were prepared according to [16] and [17] for detection of alkaloids.

Survey for the occurrence of alkaloids in the used aspergilli

Alkaloids were detected in sclerotia of tested Aspergilli by reacting with Ehrlich reagent [4,18] using a filter paper method. Four mm square agar plug is cut out from the sclerotia region of each *Aspergillus* culture separately grown on CZA. A round piece (1 cm diam.) of filter paper, Whatman No. 1, wetted with Ehrlich reagent was placed on the mycelial side of the plug. If a violet ring appears after 2-6 min, the culture contains cyclopiazonic acid or

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related alkaloids. If the reaction comes after 7-10 min, it is regarded as weak. After 10 min the violet ring will fade away. Some fungi produce alkaloids that will react with Ehrlich reagent to give pink to red or yellow rings.

Effect of different factors on the growth and weight of the sclerotia produced from *A. sclerotioniger*

A. sclerotioniger gave positive result with Ehrlich reagent more than other tested used species; so, it was chosen to optimize its growth conditions for yielding large amount of sclerotia for further work on sclerotial alkaloids.

Effect of different degrees of temperature: This experiment was designed to determine the optimum temperature for the mycelial growth and the best yield of sclerotia produced from *A. sclerotioniger* using a modified method adopted by the researchers in reference [19]. For this purpose, CZA plates were inoculated with 10 mm disc from five days old culture of *A. sclerotioniger*, then incubated at different temperature (15, 20, 25, 30, 35 and 40°C) separately. Colony diameter and the weight of sclerotia formed per plate were estimated after six days.

Effect of different pH values: This experiment was carried out using modified method adopted by the same reference. CZA medium with different pH values (4, 5, 6, 7, 8 and 9) was prepared using citrate-phosphate and boric acid-borax buffers. The plates of each pH degree were inoculated separately with 10 mm disc from five days old culture of *A. sclerotioniger*. The inoculated plates were incubated at 30°C. Colony diameter and weight of sclerotia formed per petri plate were estimated after six days. Periods (days) taken by mycelium to fill petri plate was also recorded.

Effect of different carbon and nitrogen sources: This experiment was carried out using the modified method of [20,21] using CZA medium. Sucrose was replaced in the CZA medium with equivalent amounts of carbon from five different carbon sources (fructose, galactose, glucose, lactose and maltose). Also, $NaNO_3$ was replaced by equivalent amounts of nitrogen from seven different nitrogen sources (asparagine, glycine, tryptophan, urea, $(NH_4)_2SO_4$, $NaNO_2$ and KNO_3) using the using the following equation:

X conc/L = wt. of X source (g) × Number of X atoms × X molecular weight / molecular wt. of X source

Where: X referred to carbon or nitrogen.

The pH of the medium was adjusted to 6 by citrate-phosphate buffer. The inoculated plates with A. sclerotioniger were incubated at 30°C. Colony diameter and the weight of sclerotia formed per each plate were estimated after six days. Periods (days) taken by mycelia to fill petri plate was also recorded.

Effect of carbon nitrogen (C/N) ratio: From the previous experiments, it was found that maltose and KNO_3 were the best carbon and nitrogen sources respectively for both mycelial growth and sclerotia formation. The C/N ratio was 25.5 where the carbon and nitrogen conc. in maltose and KNO_3 were 12.6 and 0.49 respectively. To study the effect of different C/N ratio on the growth and sclerotia formation of *A. sclerotioniger*, a modified method of [22] was used. Different concentrations from maltose and KNO_3 were utilized to prepare different C/N ratios (Table 1). The citrate-phosphate buffer was used in preparation of the media for obtaining PH 6. The media were sterilized in autoclave at 121°C and 1.5 atm. for 20 min. Petri plates containing uniform quantities (20 ml) of sterilized media were inoculated, incubated and treated as previously mentioned. All the studied culture conditions were considered and optimized for the production of the maximum amount of sclerotia.

TABLE 1

Carbon/Nitrogen ratio (%)

Maltose concentration (g/L)	KNO3 concentration (g/L)	C/N ratio% Calculated as C and N occurred in each corresponding source
19	5.05	13.3
23.77	4.33	16.6
21.39	2.89	22.5
30	3.56	25.5
26.14	2.89	27.5
21.39	2.16	30
23.77	1.8	40
30.9	2.16	43.33

Studies on the alkaloids extracted from sclerotia of *A. sclerotioniger*

Extraction of alkaloids: The total alkaloids of *A. sclerotioniger* were estimated [23]. Mature sclerotia were gently picked from the mycelia after 6 days incubation. Sclerotia were subsequently placed in a sieve and washed repeatedly with distilled water to remove any mycelia that remained attached with sclerotia then kept on the sieve to dry at room temperature. The alkaloids in the tested sclerotia were then extracted [16]. A random weight about 500 mg of sclerotia was powdered using a porcelain mortar and extracted by 6 ml of extraction mixture (98% methanol: water, 8:2, v/v alkalinized with ammonium hydroxide (NH_4OH). In the dark the mixture was stirred by a magnetic stirrer for 2h and then re-extracted without stirring for 12 h before filtration. The filtrate was loaded on silica gel plates for separation of alkaloids from the other components.

QUANTIFICATION ANALYSIS FOR TOTAL ALKALOIDS

Preparation of standard curve using α-ergocryptine: Standard curve was prepared using a modified method of [24]. From α-ergocryptine 5 mg were dissolved in 5 ml ethanol for preparing a stock solution of concentration 1000 µg/ml. Different dilutions (10, 20, 40, 60, 80 and 100 µg/ml] from the stock solution were then prepared. From each dilution 2 ml were added separately to 4 ml Van Urk's reagent in test tubes. Two ml ethanol was added to 4 ml Van Urk's reagent as a blank. Treatments were incubated in a water bath at 30°C for 15 min for developing the expected color indicating the presence of alkaloid. The optical density of each treatment was recorded using spectrophotometer at λ=580 nm. A standard curve was plotted using the optical density (OD) values against the known concentrations of α-ergocryptine.

Determination of total alkaloids extracted from tested sclerotia of *A. sclerotioniger*: Alkaloids were extracted from 7.5 mg of the tested sclerotia. According to the procedures modified from [24], 2 ml from this filtrate was added to 4 ml Van Urk's reagent in a test tube and 2 ml extraction mixture was added to 4 ml Van Urk's reagent in another test tube as a blank. The tubes were then incubated in a water bath at 30°C for 15 min for developing definite colors. The OD was recorded using spectrophotometer at λ=580 nm. The total alkaloids content of the tested sample was calculated using the previously prepared standard curve.

Identification of extracted alkaloid: A modified method from [25] was used for the preparation of silica gel plates which were used for the identification of the studied alkaloid. The method of [16] was used for the identification of the extracted alkaloid by thin layer chromatography (TLC). In the present work it was used for the tested alkaloid and standard alkaloids (ergocornine and α-ergocryptine); from each sample 300 µl, were spotted using automatic pipette on a line about 1.5 cm above the bottom edge of the silica plates. Each plate was then exposed to ammonia vapor for about 15 min. The plates were developed in the chromatographic jar containing the solvent system chloroform or methylene chloride - acetone (3:1) v/v for about 45 min. After complete running of the plates in the solvent system, they were sprayed by Ehrlich reagent or, examined under ultra violet irradiation at 365 nm. The retention factor (R_f), which is defined as the distance travelled by the compound divided by the distance traveled by the solvent system, was then calculated for the resulted spots.

The tested alkaloid spot was scraped from other silica plates each of spots not sprayed with Ehrlich reagent, and then dissolved in 5 ml extraction mixture. The resulted solution was centrifuged for about 10 min to discard the silica gel; this step was repeated few times to separate all the alkaloid amount from the silica. Then the supernatant was filtered through Whattman No.1 filter paper, the clear filtrate was set to evaporate the solvent to dryness to achieve the alkaloid as powder.

The tested alkaloid powder was dissolved in 5 ml from the extraction mixture solution and then analyzed using ultraviolet (UV) spectrophotometer to detect its UV spectrum in chemistry department faculty of science, Tanta University. Also, infrared (IR) spectra were carried out at room temperature using a BRUKER FT-IR spectrometer tensor 27 on the range 200 to 4000 cm^{-1} at Central Lab., Tanta University. The elemental analysis for the unknown alkaloid recognizes the percentage of carbon, hydrogen and nitrogen in the tested alkaloid sample. Thus, it was obtained by burning the solid sample in oxygenated atmosphere. This leads to turning of C, H and N to carbon dioxide (CO_2), water vapor (H_2O) and nitrogenous gases. The resulted gases were separated and the ratio of each of C, H and N was recorded. The percentage of chlorine in the alkaloid sample was also recorded. The nuclear magnetic resonance (H1NMR) analysis was carried out with 300.0687855 MHZ; the sample was dissolved in DMSO. The

molecular weight of the unknown alkaloid was achieved by mass spectral analysis using gas chromatography mass spectrometer (GC-MS). The elemental analysis, H1NMR and GC-MS were carried out at micro analytical center, Cairo University.

Statistical analysis

Statistical analysis of the present study was conducted, using the mean, standard error, standard deviation, F-value and analysis of variance (ANOVA) using the computer programme SigmaPlot 12.3.

RESULTS AND DISCUSSION

Survey for the occurrence of alkaloids in the used Aspergilli

The Ehrlich reaction method was designed to detect some indole secondary metabolites produced by fungi. The results of Ehrlich reaction are illustrated in Figure 1. It is evident that *A. sclerotioniger* produced the most obvious ring color among all the used *Aspergillus spp.* (yellow and blue) which indicated the presence of alkaloids associated with sclerotia. On the other hand, *A. melleus* gave negative results where no color was detected. Other tested Aspergilli formed yellow rings which indicated the presence of other types of alkaloids as illustrated in Figure 1.

Since the *A. sclerotioniger* revealed the best results in Ehrlich test so a large amount of its sclerotia should be obtained for extraction of alkaloids. Thus, the growth factors were of great consideration and should be studied to be optimized.

Effect of different factors on the growth and weight of the sclerotia produced from *A. sclerotioniger*

Effect of different degrees of the temperature: Table 2 illustrates the effect of different temperature degrees on the radial growth and weight of sclerotia of *A. sclerotioniger*. The different temperature degrees gave a statistically significant difference in radial growth and weight of sclerotia at $P \leq 0.001$ and $P=0.023$ respectively. The best growth at which the best weight of sclerotia was obtained when the plates were incubated at 30°C where the mean radial growth was 8.667 cm and the weight of sclerotia was 0.110 g / plate after 6 days of incubation. The lowest weight value of sclerotia was recorded at 20°C (weight= 0.00397 g/plate). The temperature degrees (15°C and 40°C) exhibited no sclerotia at all.

Effect of different pH values on the radial growth and weight of sclerotia of *A. sclerotioniger*: Table 3 represents the effect of different ranges of pH values on the radial growth and weight of sclerotia of *A. sclerotioniger*. The results indicated that the range of pH values from 4 to 7 induced the mycellial growth and formation of sclerotia. There was a significant difference ($P \leq 0.001$) between the means of radial growth and weight of sclerotia according to the effect of different pH values. PH 6 gave the highest weight of sclerotia (0.0545 g / plate) after 6 days. The lowest radial growth was obtained at PH 8 after 6 days and no sclerotia were formed, while at pH 9 the growth of the tested fungus was not detected.

Effect of different carbon sources on radial growth and weight of sclerotia of *A. sclerotioniger*: Carbon was calculated as the number of C atoms present in the used carbon sources. Table 4 showed a significant difference between the carbon sources on the radial growth and weight of sclerotia at $P \leq 0.001$. The highest radial growth (9 cm) was recorded after 6 days when the medium containing fructose as sole carbon source was used but the yield of sclerotia was relatively low (0.0107 g/plate). Maltose followed fructose as a suitable carbon source for mycelia growth recorded as 8.79 cm; however, it gave the highest weight of sclerotia (0.0283 g/plate) after 6 days. Glucose and sucrose markedly induced the radial growth and weight of sclerotia but to less extent than maltose (8.706 cm, 0.0203 g/plate and 8.16 cm, 0.0218 g/plate respectively). The media containing galactose and lactose were not favorable for growth and production of sclerotia of *A. sclerotioniger* where both results reported the lowest values.

Effect of different nitrogen sources: Nitrogen was calculated as the number of N atoms in used nitrogen sources. From Table 5, it is evident that the effect of tested nitrogen sources on the radial growth and weight of sclerotia of *A. sclerotioniger* were of statistically significant differences at $P \leq 0.001$. The inorganic sources ($NaNO_3$ and KNO_3) enhanced the mycelial growth of *A. sclerotioniger* than (NH_4)$_2SO_4$ and the organic sources (asparagine, glycine, tryptophan and urea). The radial growth was 9 cm and 8.494 cm when $NaNO_3$ and KNO_3 were used as nitrogen sources respectively. On the other hand, the highest sclerotial weight was achieved when using KNO_3 as a sole nitrogen source where the mean weight was 0.1012 g/plate after 6 days. Although $NaNO_3$ gave the best radial growth (9 cm), it gave the lowest weight of sclerotia (0.0169 g/plate) after 6 days. On the level of organic sources, asparagine recorded the best weight of sclerotia (0.0959 g/plate), while urea recorded the lowest weight (0.0263 g/plate).

Effect of carbon nitrogen (C / N) ratio on radial growth and weight of sclerotia of *A. sclerotioniger*: Each of C and N were calculated as the number of C and N atoms in each of the used sources. The effect of different C/N ratios (13.3, 16.6, 22.5, 25.5, 27.5, 30, 40 and 43.33 %) on radial growth and weight of sclerotia is illustrated in Table 6. On the media containing different C/N ratios separately the results indicated that no difference was detected in the radial growth affected by different C/N ratios where the mycelial growth was completed (9 cm) after 6 days. This means that there is
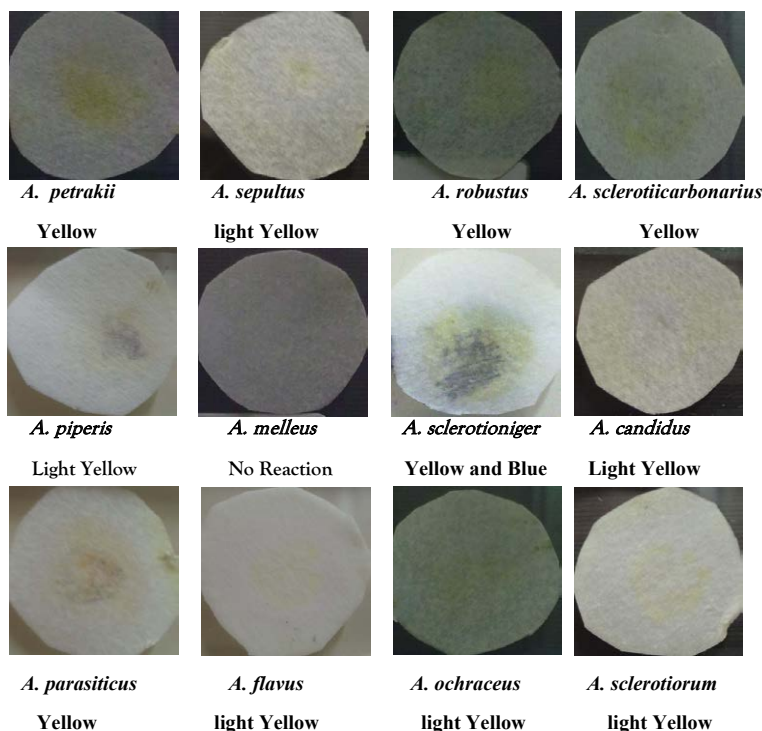

A. petrakii	*A. sepultus*	*A. robustus*	*A. sclerotiicarbonarius*
Yellow	**light Yellow**	**Yellow**	**Yellow**
A. piperis	*A. melleus*	*A. sclerotioniger*	*A. candidus*
Light Yellow	No Reaction	**Yellow and Blue**	Light Yellow
A. parasiticus	*A. flavus*	*A. ochraceus*	*A. sclerotiorum*
Yellow	**light Yellow**	**light Yellow**	**light Yellow**

Figure 1) *Occurrence of alkaloids using Ehrlich color reaction of tested Aspergilli*

TABLE 2

Effect of different temperature degrees on radial growth and weight of sclerotia of *A. sclerotioniger*

Temperature (°C)	Radial growth after 6 days (cm) (Mean ± SD)	Weight of sclerotia after 6 days (g/plate) (Mean ± SD)
15	1.65 ± 0.01	Sclerotia not observed
20	2.525 ± 0.305	0.00397 ± 0.00344
25	4.3 ± 0.265	0.0403 ± 0.0238
30	8.667 ± 0.577	0.110 ± 0.0799
35	9 ± 0	0.0405 ± 0.0351
40	4.611 ± 0.102	Sclerotia not observed
F-value	336.380*	3.993**

*SD Standard deviation; *Significant at $P \leq 0.001$; **Significant at $P = 0.023$*

TABLE 3

Effect of different pH values on the radial growth and weight of sclerotia of *A. sclerotioniger*

pH value	Radial mycelial growth after 6 days (cm) (Mean ± SD)	Weight of sclerotia after 6 days (g/plate) (Mean ± SD)
4	9 ± 0	0.0529 ± 0.0219
5	8.425 ± 0.479	0.0317 ± 0.0137
6	8.05 ± 0.52	0.0545 ± 0.0167
7	7.625 ± 0.33	0.0348 ± 0.0144
8	2.95 ± 0.58	0 ± 0
9	0 ± 0	0 ± 0
F-value	340.449*	11.794*

*SD Standard deviation; *Significant at $P \leq 0.001$*

TABLE 4

Effect of different carbon sources on radial growth and weight of sclerotia of *A. sclerotioniger* after 6 days of incubation

Carbon source	Radial growth (cm) (Mean ± SD)	Weight of sclerotia (g/plate) (Mean ± SD)
Maltose	8.793 ± 0.285	0.0283 ± 0.0116
Sucrose	8.16 ± 0.862	0.0218 ± 0.0057
lactose	6.207 ± 0.406	0.0045 ± 0.0011
Glucose	8.706 ± 0.405	0.0203 ± 0.016
Fructose	9 ± 0	0.0107 ± 0.00198
Glactose	4.79 ± 0.732	0.0099 ± 0.0027
F-value	52.425*	5.674*

*SD Standard deviation; *Significant at $P \leq 0.001$*

TABLE 5

Effect of different nitrogen sources on radial growth and weight of sclerotia of *A. sclerotioniger* after 6 days of incubation

Nitrogen source	Radial growth (cm) (Mean ± SD)	Weight of sclerotia (g/plate) (Mean ± SD)
Asparagine	6.636 ± 1.064	0.0992 ± 0.0089
Tryptophan	7.633 ± 0.153	0.0746 ± 0.0158
Glycine	7.031 ± 0.0491	0.067 ± 0.013
Urea	5.136 ± 0.627	0.0263 ± 0.0126
(NH4)2SO4	6.983 ± 0.265	0.0610 ± 0.0084
KNO3	8.494 ± 0.206	0.101 ± 0.0204
NaNO3	9 ± 0	0.0170 ± 0.0006
F-value	20.436*	15.898*

*SD Standard deviation; *Significant at $P \leq 0.001$*

TABLE 6

Effect of carbon nitrogen (C/N) ratio on radial growth and weight of sclerotia of *A. sclerotioniger*

C/N (%)	Radial growth after 6 days (cm) (Mean ± SD)	Weight of sclerotia after 6 days (g/plate) (Mean ± SD)
13.3	9 ± 0	0.142 ± 0.008
16.6	9 ± 0	0.13 ± 0.0078
22.5	9 ± 0	0.168 ± 0.0141
25.5	9 ± 0	0.167 ± 0.0031
27.5	9 ± 0	0.218 ± 0.0019
30	9 ± 0	0.166 ± 0.0068
40	9 ± 0	0.228 ± 0.0056
43.33	9 ± 0	0.245 ± 0.00028
F-value	_____	61.048*

*SD Standard deviation; *Significant at $P \leq 0.001$*

researchers, [26] recorded abundant sclerotium production at 28-32°C temperatures which support maximum growth in *A. ochraceus*. The sclerotia of *A. ochraceus* were formed at pH values ranged from 4 to 9 while there was no visible growth at pH 3 which occurred without sclerotia production at pH range of 11 to 13. While the sucrose and glutamic acid containing media as carbon and nitrogen sources exhibited the maximum number of sclerotia. Comparing to our work, earlier study found that the sclerotia were a distinguishing feature of *A. sclerotioniger* at 25°C [4].

Studies on the alkaloids extracted from sclerotia of *A. sclerotioniger*

Quantification analysis for total alkaloids content extracted from the tested sclerotia of *A. sclerotioniger* using standard curve of α-ergocryptine: The standard curve of α-ergocryptine was designated by plotting known concentrations (10, 20, 40, 60, 80 and 100 µ/ml) of α-ergocryptine in ethanol against their OD at wavelength 580 nm. From Table 7 and Figure 2 it is evident that the OD was markedly increased as the concentration of α-ergocryptine increased reaching 0.7 at 100 (µ/ml) concentrations.

The equation indicating the correlation between the values of concentration and OD was obtained from Microsoft Excel 2010 program and represented in Figure 2. The equation was:

$$Y = 0.0069 X - 0.0119$$

Where Y: OD and X: concentration.

Accordingly, the unknown concentration of total alkaloids of the extraction from 7.5 mg of sclerotia of *A. sclerotioniger* was 96.85 so, the total alkaloids resulted from one mg sclerotia was calculated to be 12.913 µ/mg.

Identification of extracted alkaloid: When spraying with Ehrlich reagent the extracted alkaloid from sclerotia of *A. sclerotioniger* was detected using the silica gel plates as blue band while the standard alkaloids (ergocornine and α-ergocryptine) revealed violet color bands (Figure 3A). In the meantime, the extracted alkaloid was also detected as blue florescent spots on silica gel

statistically insignificant in radial growth values in response to different C/N ratios. However, the highest weight of sclerotia was recorded when using C/N ratio of 43.33 followed by C/N ratio 40 and 27.5% where the weights of sclerotia were 0.245, 0.228 and 0.218 g/plate respectively. There was a significant difference between the C/N ratios on the weight of sclerotia at $P \leq 0.001$.

All the obtained results in optimizing the culture growth conditions for the sclerotia formation of *A. sclerotioniger* were in accordance with those of other workers [20] on *Aspergillus caelatus* where the optimal synnema/sclerotium formation occurred between 28°C–30°C, pH 6 and 10. Also the same author reported that the mono-saccharides (arabinose, dextrose and xylose) and the disaccharides (cellobiose, melibiose, sucrose and trehalose) stimulated the most synnema/ sclerotium formation in *A. caelatus* isolate (NRRL 25528). He also stated that serine, threonine, potassium nitrate and sodium nitrate induced most of sclerotium formation in *A. caelatus* NRRL 25528. But in contrast to our results he found that the maximum production for both synnemata and sclerotia by the *A. caelatus* isolates NRRL 25528 and NRRL 26119 occurred at C/N ratio of 8.6. On the other hand, other

TABLE 3OD of different concentrations of α-ergocryptine in ethanol at 580 nm

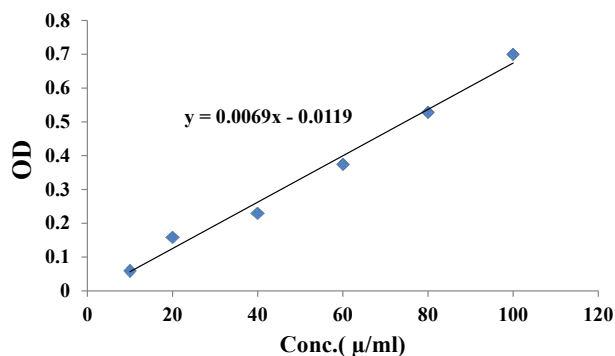| Conc. of α-ergocryptine (µ/ml)	OD
10	0.06
20	0.16
40	0.23
60	0.375
80	0.53
100	0.7

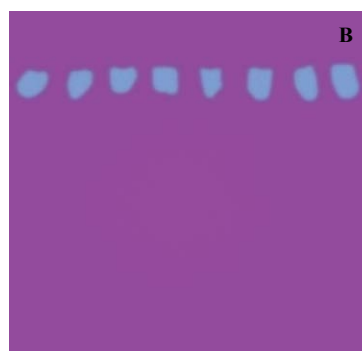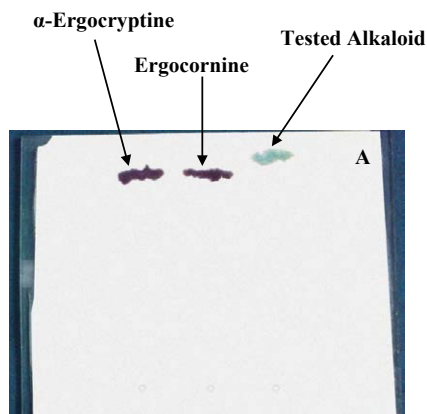**Figure 2)** *Standard curve of α-ergocryptine in ethanol*

Figure 3) (A) *The colored bands of ergocornine, α-ergocryptine and the tested alkaloid extracted from sclerotia of A. sclerotioniger on silica gel plates after spraying with Ehrlich reagent. (B) The blue florescent spots of the tested alkaloid extracted from sclerotia of A. sclerotioniger under UV lamp at 365 nm.*
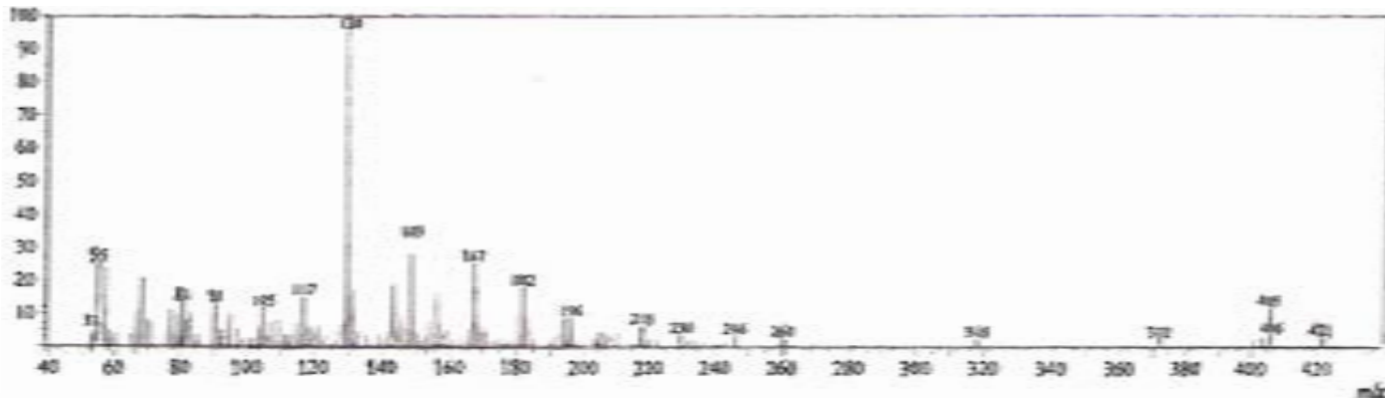

Figure 4) *The GC mass spectrum chart of the extracted alkaloid from sclerotia of A. sclerotioniger*

plates when tested with UV irradiation at 365 nm (Figure 3B). The blue color of tested alkaloid indicated that this alkaloid belongs to indole alkaloids as mentioned by [27]. So that the chemical structure of the tested alkaloid was expected to contain the following indole ring:
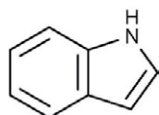
The R_f is defined as the distance reached by the compound divided by the distance reached by the solvent system. For the tested alkaloid R_f was found to be 0.976 while R_f of ergocornine and α-ergocryptine were 0.696 and 0.72 respectively.

The above results indicated that the extracted alkaloid belongs to a group of alkaloids named indole alkaloids. Many reports revealed that sclerotia of some species of *Aspergillus* section nigri produce indole alkaloids. The black fungus, *Aspergillus indologenus* was isolated from a soil in India which produced the insecticidal compounds okaramins A, B, H, and two types of indol-alkaloids which have not been elucidated [28]. The same

author also reported that A. *violaceofuscus* was found to be related to A. *japonicus*, and produced some of the same interesting indol-alkaloids as A. *indologenus*. While, A. *sclerotiicarbonarius* which is a biseriate species similar to A. *carbonarius* and A. *ibericus*, produces abundant sclerotia and some unique indol-alkaloids [29].

The UV spectrum analysis of the tested alkaloid sample was found between 276 and 283 nm. Also, the percentages of carbon, hydrogen, nitrogen and chlorine were found to be 76.47, 10.24, 2.67 and 15.61 % respectively.

The molecular weight of the unknown extracted alkaloid was recorded using GC mass spectrum analysis. The molecular weight of the tested alkaloid was found to be 421 (Figure 4).

Figures 5 and 6 illustrated the H1NMR and IR analysis charts for the tested alkaloid respectively. These charts indicated the presence of OCH_3, CH_2, CH_3, OH, C=O, NH or NH_2 groups and aromatic system in its structure.

From all the previously mentioned analysis it was obvious that the extracted alkaloid from the sclerotia of A. *sclerotioniger* may be predicted as
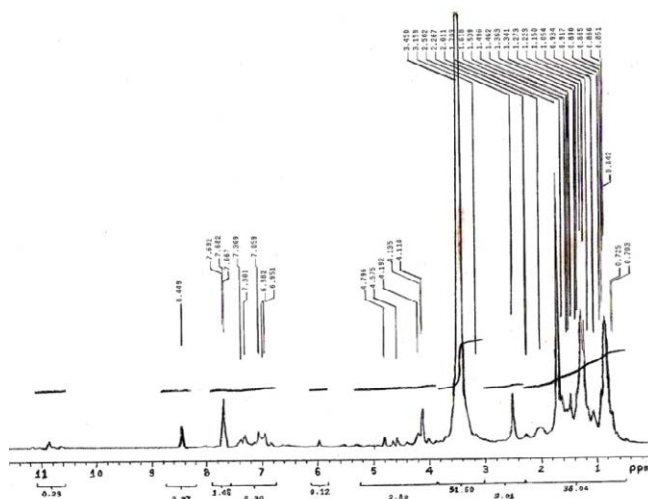
Figure 5) *The H1NMR chart of the extracted alkaloid from sclerotia of A. sclerotioniger*
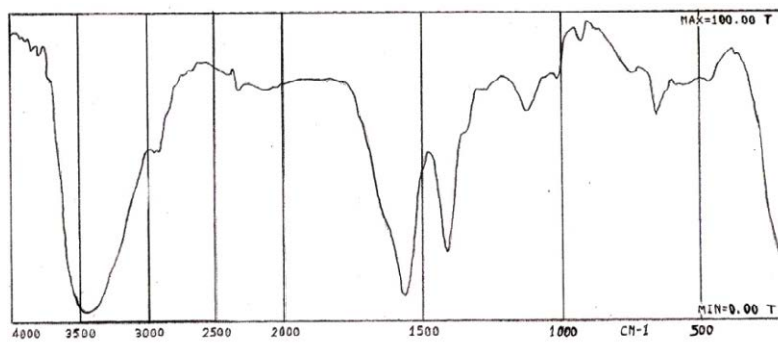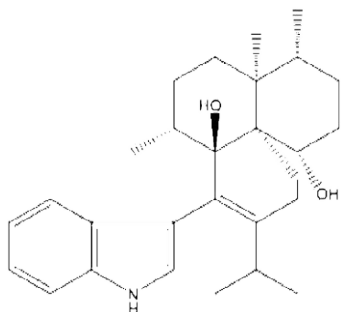

Figure 6) *The IR chart of the extracted alkaloid from sclerotia of A. sclerotioniger*

a chloro derivative from Aflavinine compound. The chemical structure of Aflavinine could be designed as follow:

This finding was supported by earlier study [13] who studied different species of *A. niger* group and stated that all the tested strains of the black aspergilli produced a large number of known and as yet unknown extrolites. Many of these extrolites belonged to aflavinines. They reported that *A. costaricaensis* and *A. piperis* produce 14-epi-14-hydroxy 10,23-dihydro-24,25 dehydroaflavinine and 10,23-dihydro-24,25-dehydroflavinine. Also, [30] found that *A. tubingensis* produced:

- 14-epi-14-hydroxy-10,23dihydro-24,25-dehydroaflavinine,

- 10,23-dihy-dro24,25 dehydroaflavinine and

- 10,23-dihydro-24,25dehydro-21-oxo-aflavinine.

CONCLUSION

From the present study it could be concluded that, the best radial growth and weight of sclerotia of *A. sclerotioniger* were obtained when the pH of used CZA medium was adjusted at pH 6 while the best incubation temperature was 30°C. Maltose and KNO₃ recorded the best radial growth and weight of sclerotia of *A. sclerotioniger* when used as carbon and nitrogen sources in CZA medium at C/N ratio 43.33%. The resulted sclerotia of *A. sclerotioniger* were

tested for the production and identification of alkaloids where the quantity of total alkaloids content resulted was equals 12.913 µ/ml. The blue color of tested alkaloid on TLC when sprayed with Ehrlich reagent indicated that this alkaloid belongs to indole alkaloids. Also, according to the CHN percentage, UV spectrum, GC mass spectrum, H1NMR and IR analyses, the extracted alkaloid from the sclerotia of *A. sclerotioniger* was predicted to be a chloro derivative of aflavinine.

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