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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *ASPERGILLUS FLAVUS* CLINICAL ISOLATES FROM HUMAN AND SHEEP SPUTUM

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ABSTRACT : ***Aspergillus flavus*** **is one of most important and ubiquitous fungal filamentous pathogen for animals, humans and plants. It is widely distributed in soils, air, water and other environmental compartments making it a serious opportunistic fungal pathogen. Regardless its toxic secondary metabolites, in particular Aflatoxin B1, a carcinogenic mycotoxin, it is considered a main cause of invasive aspergillosis in all animals, poultry and a second main cause in humans after** ***Aspergillus fumigatus*****. In this study, we worked to isolation andidentificationof this type of** ***Aspergillus*** **species from sheep and human's sputum and then characterization of their ability to produce AFB1. We were collected 150 samples from humans and 150 samplesfrom sheep. The results of macroscopic examination on general media such asSabouraud dextrose Agar (SDA) and potato dextrose Agar (PDA)were matched with results of microscopic examination, where they showed 14 of 150 (9.33%) positive samples of*** **Aspergillus flavus** **for human samples, while 25 of 150 (16.66%) for sheep samples. The results of aflatoxigenicity on differential media showed 10 0f 14 (71.42%) for human samples and 19 of 25 (76%) for sheep samples on yeast extract sucrose (YES) media, while 8 of 14 (57.14%) for human samples and 17 of 25 (68%) for sheep samples on coconut Agar media (CAM). We conclude that this fungal pathogen and their secondary metabolites constitute a dangerous problem for humans and animals, therefor it is should be diminished in our environments. Also, the test on (CAM) with UV light was more accurate than (YES) with ammonia vapor.**

Key words : Samples, *A. flavus*, isolation & aflatoxigenicity.

INTRODUCTION

Aspergilli are the most common secondary pathogen of fungal invasions and in hospital settings, with of nearly five person of (100, 000) in the USA (Wilson *et al*, 2002 and Vermeulen *et al*, 2014). *Aspergillus* forms asexual spores (conidia) which spread through the environmental air. When these spores inhaled with aerosol, this mostly considers the main rout of infection (Weigt *et al*, 2013). *Aspergillus* fungi are valuable pathogen for immunocompromised patients in both morbidity and mortality (Hamzah and Hasso, 2019). The common infectious mortality in patient of bone morrow transplant stem cell is invasive aspergillosis and serious cause of disseminated aspergillosis in other imunosupressed persons. It is may induce chronic, allergic and saprophytic disorders (Walsh *et al*, 2008 and Yoshida *et al*, 2015). Minnat and Khalaf (2019) were recorded (25.9%) of *A. flavus* as cause of canine dermatomycosis among other *Aspergillus* species isolated from skin of doges.

Aspergillus flavus is saprophytic soil filamentous mold that infect and contaminate the growing and harvested crops with carcinogenic mycotoxin (aflatoxin). It is also infect humans and animals resulting in aspergillosis particularly in immunocompromised populations (npkeller@wisc.edu), so, support immune system against the oxidative stress may be used (Twegh *et al*, 2020). *A. flavus* and *A. parasiticus* often the widely assessed food decayer molds, after the discovery of toxic and carcinogenic metabolites in 1960s (Aflatoxin) (Pettersson and Leong, 2011). Several studies about fungal toxin contamination and investigations in medicinally therapeutic herbs and associated products revealed that aflatoxins (AFs) and other important mycotoxin are the most common contaminants (Diana *et al*, 2009 and Han *et al*, 2012).

Microscopically, *A. flavus* chonidiophore has rough stipes about (400µm-1mm) or more in length, ended with spherical vesicle of 20 to 50µm in diameter, carrying both metulae and phialides of (7-10µm) long. The conidia are slightly roughened and spherical (3-5µm) in diameter (French Agency for Food, 2012).

MATERIALS AND METHODS

Sample collection

The target samplewas the sputum from patient (sheep and humans) with respiratory diseases (Shrimali *et al*, 2013), which collected from ages between 14-80 years old including both genders, male and female (Al-Zuhairy, 2018). 300 samples were collected from both human in (Consultation center of respiratory diseases in misan province) and animals (sheep) with respiratory problems from (Slaughter house in misan) using sterile transport media swaps. After, the samples directly transport to microbiological laboratory to be microscopically examined and cultured on general and differential media.

Direct examination

Samples were directly examined by microscope using KOH wet slides (Shrimali *et al*, 2013).

Indirect examination

Samples of sputum were cultured on two general fungal media Sabouraud dextrose Agar (SDA) (Himedia, India) and potato dextrose Agar (PDA) (Himedia, India), and two differential media (Malt extract Agar (MEA) (Himedia, India)and Czapek Doxe media (CZA) (Oxoid, England), which werepurchased as standard media, at PH 6.5 and temperature 37ºC (Lahouar *et al*, 2016) for 5-7 days with daily observation. Detection of their aflatoxigenicity was performed on other two media; Coconut agar media (CAM) and yeast extract sucrose agar (YES) media, which were locally prepared as following:

Coconut agar media (CAM) : The media was prepared depending on method of Davis *et al* (1987) with slight modification. The crude coconut was locally, purchased). Hashed coconut (100g) homogenized with 200ml of heated distilled water about 10 minutes. The mixture was filtered by 4 cloth layers from cheese cloth and the pH was adjusted to pH:7, then added the agar by 20g/L and the media then boiled and cooled to 45^0C. The pH was adjusted again to pH:7 and the media then steriled autoclaved at 15 Ib/in^2 for 20 min. After, decreased its temperature to 45^0C and poured in to petri sterile dishes.

Yeast extracts sucrose agar (YES) media : It is prepared according on procedure of Khaddor *et al* (2007) with some modification. 2% of yeast extract, 20% sucrose and 2g agar mixed in 100ml distilled water and heated at boiling degree and cooled adjusted pH to 7 and then autoclavedat 15 Ib/in^2 for 20 min, then poured in to sterile dishes.

The following observations were evaluated during daily examination depending on criteria of Maza *et al* (1997 and Ellis *et al* (2007) with some modification.

1. Morphology of growth (color and consistency) on different media.

2. Reverse side color (changed with time) on different media.

3. Microscopic characterization (shape and size) Lactophenol cotton blue (LCB) stain.

Aflatoxigenicity exanimation

This culture method was performed depending on procedures of other scientific researches (Rodrigues *et al*, 2007 and Thathana *et al*, 2017) on coconut agar media (CAM) and (Saito Machida, 1999 and Shekhar *et al*, 2017) on yeast extract sucrose agar media (YES). Where after culturing on (CAM) at 28 for 7days, the cultures were examined on UV light plate (365nm) wave length, while the cultures on (YES) at 28 for 7days were examined by pouring of two drops of ammonium hydroxide on lids of inverted petri dishes to be exposed to ammonia vapor and put them in incubator for 1-2 hr with observation the color changes.

RESULTS

Isolation and identification of *A. flavus*

14 out of 150 positive samples (9.33%) of *A. flavus* isolates were recorded from human sputum samples and 25 out of 150 were positive samples (16.66%) from sheep sputum samples (Table 1).

Table 1 : Shows numbers of collected samples and percentage of positive samples in macro and microscopic examinations of *A. flavus.*

Parameters	Sheep	Humans
Number of samples	150	150
Macroscopic examinations	25 (16.66%)	14 (9.33%)
Microscopic examinations	25 (16.66%)	14 (9.33%)

Macroscopic identification

Culturing on general media : The samples were cultured on general fungal media such as potato dextrose agar (PDA) and sabouraud dextrose agar (SDA) at 37ºC for 5-7 days. The face of colonies of *A. flavus* were rounded, velvety surface, wet and yellowish-green in color with clear white edgeon these media (Fig. 1). Further, the reverse color of colony appears as hyaline or yellowish-brown and changed with time.

Culturing on differential media : *A. flavus* isolates were identified morphologically on specific differential media according to color of colonies.

Culture of *A. flavus* on malt extract agar (MEA) media : The colony of *A. flavus* on malt extract agar (MEA) at 37ºC for five days appears as dark green in

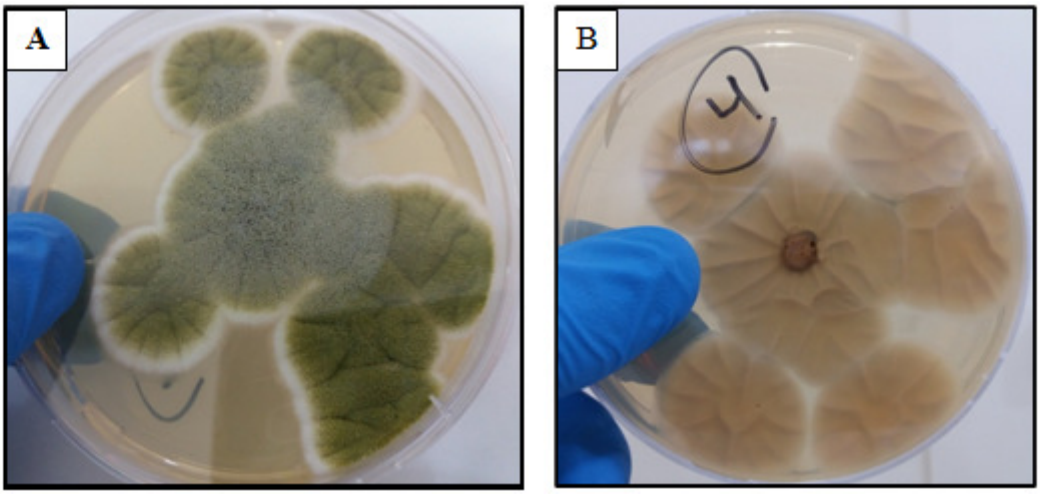

Fig. 1 : Shows *A.flavus* on sabouraud dextrose agar for 5 days at 37 ºC. The face of colony appears rounded, white edge and yellowish-green in color and velvety surface. **B.** Shows the yellowish to hyaline reverse face.

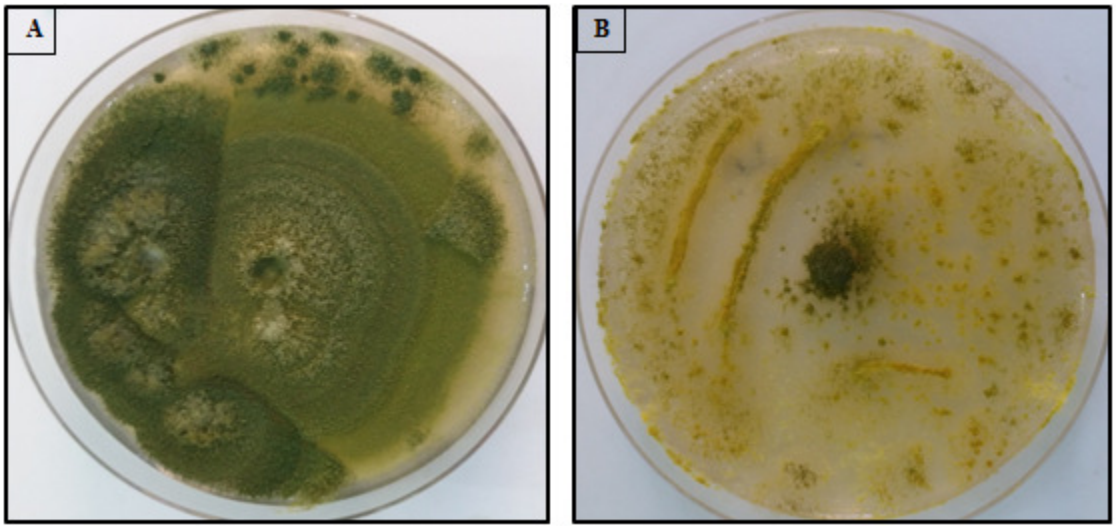

Fig. 2 : A. The *A. flavus* isolate colony on malt extract agar, at 37 ºC for 5 days, appears with deep or dark green in color of its top surface. **B**. Colony of *A. flavus* isolates grew on (CZA) at 37 ºC for 5 days appears with scattered yellow color of top surface without exudate.

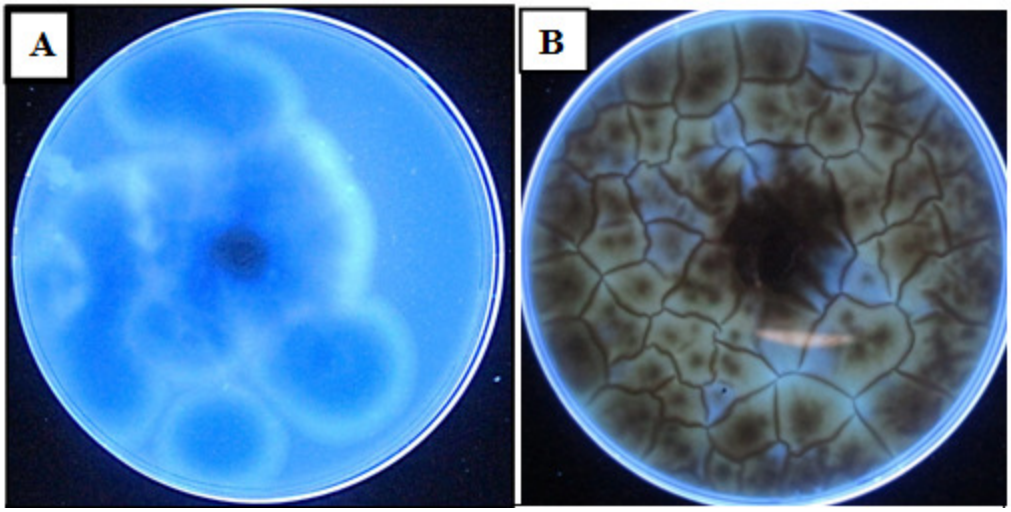

Fig. 3 : Illustrates the greenish-blue color border of *A. flavus* colony on (CAM) at 28ºC for 7 days indicating the aflatoxin-producing strain (**A**) compared with the control one (**B**).

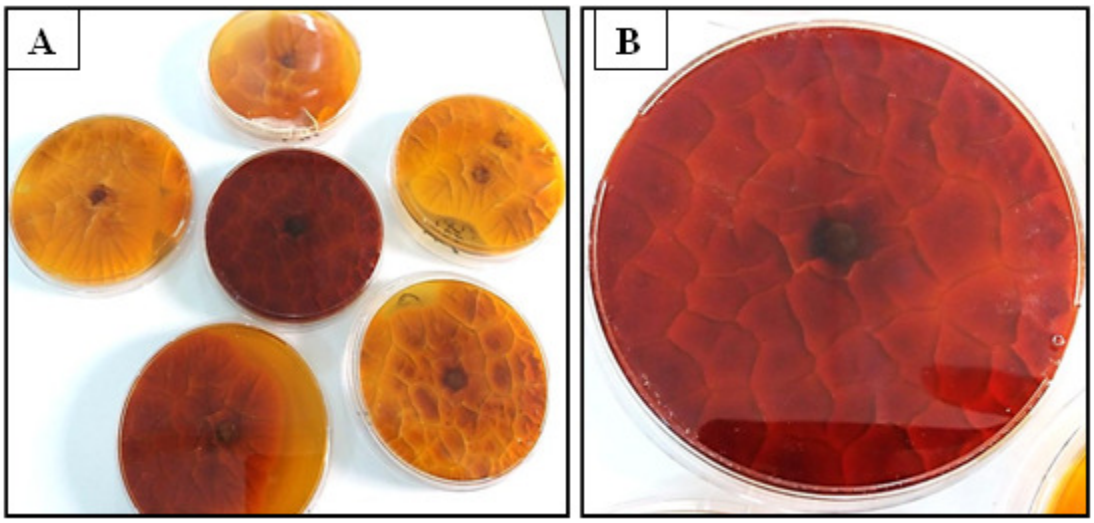

Fig. 4 : Illustrate the orang to plum-red color of reverse side of *A. flavus* colony on (YES) at 28 ºC for 7 days indicating the aflatoxin-producing strain. The red-plum is high concentration, red color is concentrated, pink or orange less concentration and yellow or creamy very low or no concentration (Shekhar *et al*, 2017).

color with white edge, while the reverse color was pale yellow or hyaline (Fig. 2A).

Culture of *A. flavus* on Czapek agar (CZA) media : *A. flavus* colonies on Czapek agar (CZA) media at 37ºC for five days were characterized by scattered growth, yellow in color on top surface without exudate (Fig. 2B).

Culture of *A. flavus* on aflatoxigenic differential media : The colonies of *A. flavus* isolates also were characterized morphologically by their ability to produce aflatoxins on specific media to confirm the diagnosis.

A. Culture of *A. flavus* on coconut agar media (CAM)

A. flavus isolates were cultured on coconut agar media (CAM) for 7 days at 28ºC and then the colonies in petri dishes examined on ultraviolet source at 365nm. The detection of aflatoxigenicity was depending on emission greenish-blue fluorescent light around reverse sides of colonies (Fig. 3). The results showed 8 of 14 (57.14%) for human samples and 17 of 25 (68%) for sheep sample were positive for this test (Table 2).

B. Culture of *A. flavus* isolates on yeast extract sucrose agar (YES) media

After growing of *A. flavus* on (YES) media for 7 days at 28ºC, the petri dishes were inverted and then poured 2 drops of ammonium hydroxide on their lids and incubated again allowing reacting their metabolites with ammonia vapor. With the time the reverse sides color were changed from pale yellow to orang or plum-red according to concentration of aflatoxin (Fig. 4). 10 of 14 (71.42%) for human samples and 19 of 25 (76%) for sheep samples were positive for this test (Table 2).

Table 2 : Shows percentages of positive cultures for *A. flavus* on CAM and YES.

Parameters	In Sheep	In Humans
Culture on CAM	17 of 25 (68%)	8 of 14 (57.14%)
Culture on YES	19 of 25 (76%)	10 of 14 (71.42%)

Microscopic identification

The results of microscopic examination was matched with results of macroscopic examination on general fungal media (16.66%) & (9.33%) for sheep and humans respectively (Table 1). *A. flavus* appeared under light microscope by using lactophenol cotton blue (LCB) as branching mycelia consisting from septate hyphae and vesicle bearing conidiophores characterized by thick uncolored wall. The vesicles are globose or subglobose in shape covered with uniseriate or biseriate phialides. The spherical conidia arranged on the phialides characterized by thin and mildly roughened walls (Fig. 5 A, B, C & D).

DISCUSSION

In our study, the high percentage of *A. flavus* isolates from animals samples (16.66%) compared with humans samples (9.33%) may due to bad hygiene and management (Elad and Segal, 2018). This percentage was less than that obtained by Diba *et al* (2007), (55%) *A. flavus* of (52) clinical isolates and this may due to differences in weathers and environmental contamination. Also, the result was nearly agreed with Al-Zuhairy (2018), while the results was high than that presented by Shrimali *et al* (2013), (3 of 61) isolates from clinical samples (sputum) in humans, which due to the same mentioned cause. These differences in percentages may attribute

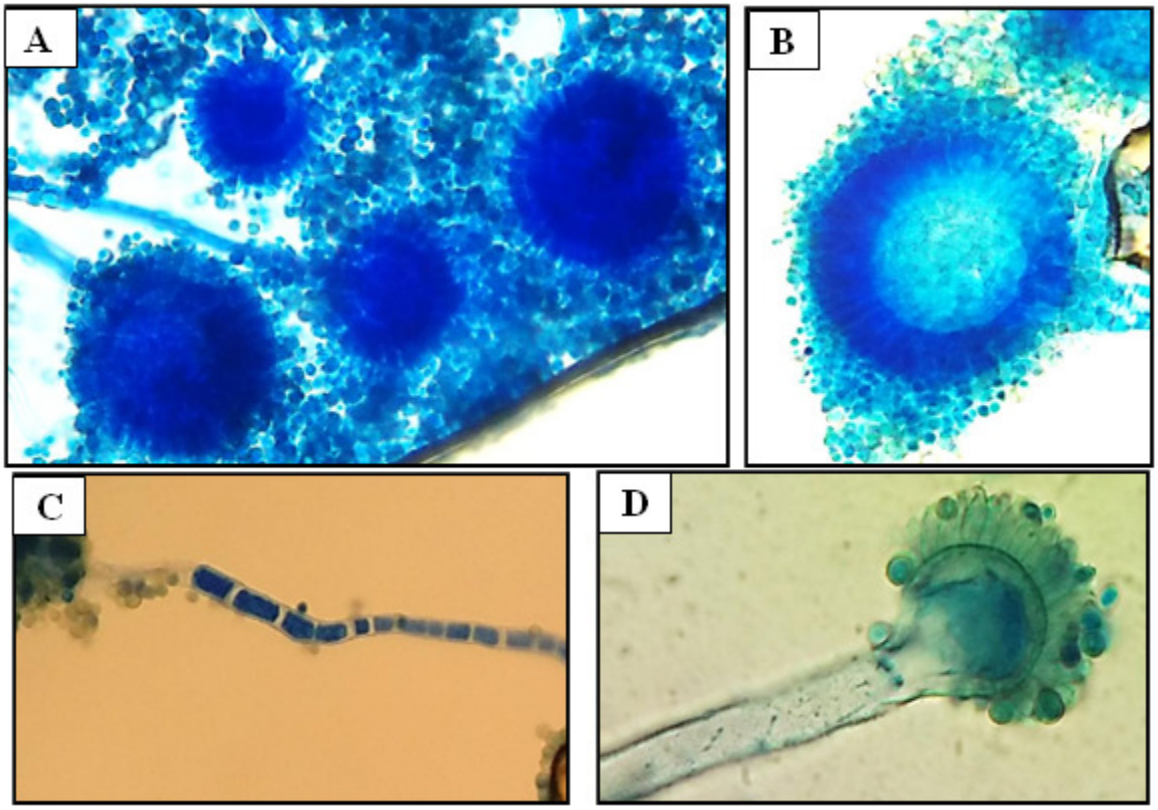

Fig. 5 : Illustrate the microscopic appearance of *A. flavus*. Four conidiophores with conidial heads surrounded with high number of conidia **(A)**. Vesicle surrounded by biseriate phialides **(B)**. Prominent septate hyphae and conidiophore **(C)**. Conidiophore and vesicle covered by uniseriate phialides **(D)**.

to environmental variation of countries where the studies were performed. The velvety yellowish-green color of colony with white border on PDA and SDA (Fig. 1) was similar to results of Diba *et al* (2007), Gonu *et al* (2015), Thathana *et al* (2017) and Olokkaran *et al* (2019). The yellow or brown color of its reverse side in present study also presented previously by Gonu *et al* (2015) and Bharose *et al* (2017).

The deep or dark green color of *A. flavus* colony on MEA (Fig. 2A) was as found by other researches (Afzal *et al*, 2013; Thathana *et al*, 2017; Zulkifli and Zakaria, 2017). The scattered yellow growth of isolates on CZA without exudate (Fig. 2A), these results were agreed with other researcher's results such as Nyongesa *et al* (2015), Thilagam *et al* (2016). The differences in the mycelial mat colors in all mentioned media media may attribute to variation in the number of secondary metabolic components secreted by *A. flavus* on different culture media (Nayak *et al*, 2018).

The greenish-blue (fluorescent) color around fungal colony on CAM (differential media) after that appeared on UV light plate (Fig. 3A) was represent aflatoxigenicity of *A. flavus* islotes and this resemble to results gained by Rodrigues *et al* (2007), Alkhersan *et al* (2016), Thathana *et al* (2017). Moreover, this differential method has high efficacy compared with other methods, where the false positive is (0%) and this consistent with results of Ismail *et al* (2016), Monda *et al* (2020) and Rao *et al* (2020). The principles of this test depend on spectra of absorbance and emission of aflatoxins, where the high absorbance at 360 nm of UV light (Fallah *et al*, 2011).

Appearing of Red-plum color of reverse side of *A. flavus* colony on YES after exposure to ammonia vapor (Fig. 4) was compatible with previous results of Saito and Machida (1999), Sudini *et al* (2015), Alkhersan *et al* (2016), Moradi *et al* (2017) and Shekhar *et al* (2017), where the efficacy of this differential method (89.64%) is less than in CAM in positivity (Kushiro *et al*, 2018; Rao *et al*, 2020). The color changes due to alteration in PH of intermediates chemical compounds formed during aflatoxin synthesis which affected by ammonia (Rao *et al*, 2020). The biochemical bases of this conversion (to Plum-red) was investigated by Abbas *et al* (2004), where they tested the methanol extracted-secondary metabolites from culture of *A. flavus* on (PDA). They found that this extracts was contained yellow pigments which turned in to plum-red color when mixed with any base particularly (ammonium hydroxide), in other hand, new addition of acids will change them in to the original yellow color. They conclude that these pigments play as PH marker

dyes.

The first scientists who correlated these pigments with aflatoxin production were (Lin and dianese, 1976). The brilliant yellow dyes had been called anthraquinone dyes which considered as intermediate substances in aflatoxin bio-formation and production pathways (Bhatnagar *et al*, 2003; Shier *et al*, 2005).

The microscopic features of *A. flavus*, which stained with (LCB), (Fig. 5) in this study and viewed under light microscope were in consistence with the information and features mentioned in global fungal text books and atlases such as Howard and Dekker (2002), Samanta (2015) and researches of Seelan (2004), Rodrigues *et al* (2007), Gautam and Bhadauria (2012), Afzal *et al* (2013), Thilagam *et al* (2016), Thathana *et al* (2017), Zulkifli and Zakaria (2017), Olokkaran *et al* (2019).

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