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A contribution on the pathogenicity of *Fusarium oxysporum* isolated from cultured Nile tilapia (*Oreochromis niloticus*) with trials for the treatment

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ABSTRACT

Fusarium oxysporum is one of the systemic mycosis that affects fish. This study was designated to determine the pathogenicity of *Fusarium oxysporum* in *Oreochromis niloticus* with a clinical trial for treatment. Fusarium oxysporum isolated from naturally infected *Oreochromis niloticus* collected from different fish farms at different governorates in Egypt namely, El Fayum, kafr El-sheikh and Alexandria. Fish samples with a total number of 100 were collected during the period from May to October 2019. Microbial examination revealed infection with *Fusarium oxysporum* that was identified by molecular sequence analysis of the internal transcript spacer (ITS) region of their ribosomal RNA gene. The confirmed isolate of *Fusarium oxysporum,* was used in experimental infection to determine its Pathogenicity followed by a trial for treatment of clinically affected fish with Curcumin and H2o2. The clinical signs associated with experimental infection were exophthalmia, abnormal swimming behaviour, granuloma in different internal organs including gills, liver and spleen with enlargement of spleen and congestion of liver. Examination revealed severe pathological alteration gills, liver and spleen manifested by severe congestion of the central venous sinuses of the primary gill lamellae as well as the blood vessels of the secondary lamellae associated with lamellar edema , congestion of hepatoportal blood vessels and sinusoids associated with perivascular edema and presence of variable granulomas embedded in the splenic parenchyma . Curcumin and H_2o_2 showed a mild beneficial effect as a treatment for *Fusarium oxysporum* infection in cultured *Oreochromis niloticus*.

INTRODUCTION

Fusarium genus includes a wide range of species, commonly found in plants, air, soil, marine water and freshwater (Michielse and Rep, 2009). *Fusarium* infections have been recorded in many aquatic animals such as sharks, dolphins, whales, shrimp, as well as

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reptiles and amphipians (Salter et al.,2012). Because of growth of aquaculture, fish have exposed to many fungal agents due to stressful conditions of fish farming (Yanong,2003). Indeed, fungal problems in fish usually occur when fish exposed to change in some water parameters such as temperature and salinity, or microbial problem as parasite and bacteria. *Saprolegnia* species beside some aquatic fungi frequently lead to some problems in cultured and wild freshwater fish. Also, *Fusarium* and *Aphanomyces* are known as significant fish pathogens affecting shell fish and marine fish (Hatai ,2012). It has been noticed that marine fish that infected with Fusarium fungi, showed deep, lesions in eye and skin with severe ulceration and necrohemorrhagic dermatitis (Yanong,2003). Kumari and Kumar (2015) concluded that fusarium species were among the most common mycotic isolates infecting freshwater fishes in Gandak River in India. Nile tilapia (*Oreochromis niloticus*) is one of the most frequently cultivated species in the world as it has high protein content, fast growth and good tastiness. Cutuli *et al*. (2015) confirmed the pathogenicity of F. oxysporum in Nile tilapia (O. niloticus) where experimental challenge with F. oxysporum has been successfully developed into infection. Curcumin, is a yellow, acidic, phenolic turmeric herb extract . It is produced mainly in China ,india, japan and other tropical and subtropical countries. In recent years, studies have shown that dietary curcumin has a broad range of pharmacological effects, including the reduction of free radicals(Toda *et al*., 1985), antioxidants activity (Ruby *et al*., 1995), antibacterial and immune stimulant effects (Ganguly *et al*., 2010).Hydrogen peroxide (H_2O_2) was used against many known pathogens in aquaculture, including parasites(Powell and Clark ,2004), fungi (Rach *et al*., 2004) and bacteria (Gaikowski *et al*., 2003). Hydrogen peroxide is active against fungi, yeast and viruses and has an effective role against gram-negative bacteria than against gram-positive bacteria in general. Some pathogens as anaerobic bacteria are more susceptible because they lack e catalase and superoxide dismutase, which break the peroxide compound (Imlay, 2003). So this study was planned to study the pathogenicity of *fusarium oxysporum* isolated from naturally infected *Oreochromis niloticus* with attempt of clinical trial for treatment.

MATERIALS AND METHODS

1. **Fish samples:**a total number of one hundred cultured Nile tilapia (*Oreochromis niloticus*) of different body weight ranged from130 - 160 g. Fish were collected from private fish farms from various localities in Egypt (El fayoum,kafr El-sheikh and Alexandria) displaying clinical signs. The samples were immediately transported in ice tank to the laboratory of department of aquatic animal medicine and management, faculty of veterinary medicine, Cairo University.
2. **Gross clinical examination:** Naturally, infected fishes have been clinically examined to identify any clinical anomalies (Austin and Austin, 1987).
3. **Post mortem (PM) examination:** Freshly dead fish specimen has been examined for any gross pathological lesions (Plumb and Bowser ,1982).

4. **Mycological examination:** Fish was disinfected with a 70 % ethyl alcohol. Gills, spleen, liver, and kidneys of freshly sacrificed fish were collected under full aseptic conditions and inoculated into Sabouraud's dextrose agar medium (SDA)supplemented with 0.05 mg / L chloramphenicol (Whitman,2004). The plates were incubated at 25-28°C for 3-5 days. The plates without growth were not discarded until 2 weeks (Feingold and Baron ,1986). Pure cultures were obtained by sub culturing the positive plates on Sabouraud's dextrose agar plates and reincubated at 25 - 28°C for 3-5 days and examined for macro- and micromorphological characteristics (Collins and Lyne, 1984).
5. **Morphological identification of the mould:** Pure cultures were examined for gross and micro-morphological characteristics according to Frey *et al*.(1979).
   - **Macroscopical examination:** During macro morphological investigation, growth rate, texture, difference in color during growth, final color of the surface and reverse sides of the colonies and final colour has been observed and recorded
   - **Microscopical examination (Solutip method):** For micro-morphological studies, a small portion of a fresh colony's periphery was picked up using a piece of solutip's sticking surface and placed on a clean slide with a drop of lactophenol blue stain(Cruickshank *et al*.,1982) and microscopically examined. Microscopic examination was performed to show septation of hyphae, roughness or smoothness of conidiophores, the vesicle's shape, number and arrangement of the stigmata's rows.
6. **DNA extraction and PCR assay:** After 3 freeze−thaw cycles, the genomic DNA of fungal samples was extracted using a commercial DNA plant extraction kit (Thermo Scientific). Extracted DNA was visualized by electrophoresis of the agarose gel (0.9 %) until stored at −20 ° C. The universal primer pair for the internal transcribed spacer (ITS) was used for the identification of fungal species; primary sequences were ITS1 (5'-TCC GTA GGT GCT GCG G-3) 'and ITS4 (5'-TCC TCC GCT TAT TAT GC-3') (White et al.,1990) . DNA amplification was performed in a thermocycler (MJ Mini, Bio-Rad) The sizes of the PCR product was visulaized by electrophrases (SinaClon, Iran).
   - **ITS rDNA sequence analysis:** ITS 'PCR- products for the fungal isolate were directly sequenced by Big Dye terminator sequencing (Applied Biosystems) in both directions on ABI 3730 machines. To provide the finalized ITS sequence the forward and reverse directions of the read sequence were used. The sequence obtained for the ITS was compared using the BLAST method to relative existing sequences in GenBank. (Sneath & Sokal 1973, Tamura *et al*. 2007).
7. **Histopathological examination:** Different tissue specimens from liver, spleen, gills and kidneys of naturally infected *Oreochromis niloticus* were cut and fixed in 10% neutral buffered formalin. The sections were then routinely processed and embedded

in paraffin blocks, cut into 5µm thick sections and stained with H&E for routine histopathological examination (Roberts ,2001).

8. Experimental design:

- **Inoculum preparation:** Fungal strains of *F. oxysporum* cultured on SDA at 25°C for 7 days, and then conidial mass was harvested by adding 20 ml sterile distilled water into each culture plate, followed by collection of the suspension in 30 ml. sterile autoclavable tubes. Suspensions were filtered through two layers of sterile medical gauze to ensure that filtrate contain fungal, concentrations of which were calculated using an erythrocyte counting chamber and adjusted to 1×10^4 conidia ml in sterile distilled water (Hassan and Selim, 1983)
- **Experimental design:** A total number of 80 *Oreochromis niloticus* for experimental design were divided into four groups of 20 fish per group:

The first group (negative control): represents normal untreated fish. The Second Group (control positive): represents fish experimentally infected with *Fusarium oxysporum* and not treated. The third group : represents experimentally infected fish ~~and~~ treated with curcumin 4mg/l water as soon as the appearance of clinical signs according to Liu et al.(2017).The fourth group: represents experimentally infected fish and treated with H_2O_2 4mg/l water(bath rout for one hour) as soon as the appearance of clinical signs according to Russo *et al*. (2007)

RESULTS

1. **Clinical and post mortem examination:** naturally infected *Oreochromis niloticus* showed different lesions such as erosion of gills, erosion of fins, congestion of liver and kidney as well as enlargement and congestion of spleen **(Fig. 1**)

2. **Morphology:** Colonies were fast growing, floccose, and white to and produced only in false heads. Macroconidia were slightly sickle-shaped, up to 3(-4)-septate, and with an attenuated apical cell and a pedicellate basal cell (Fig. 2).

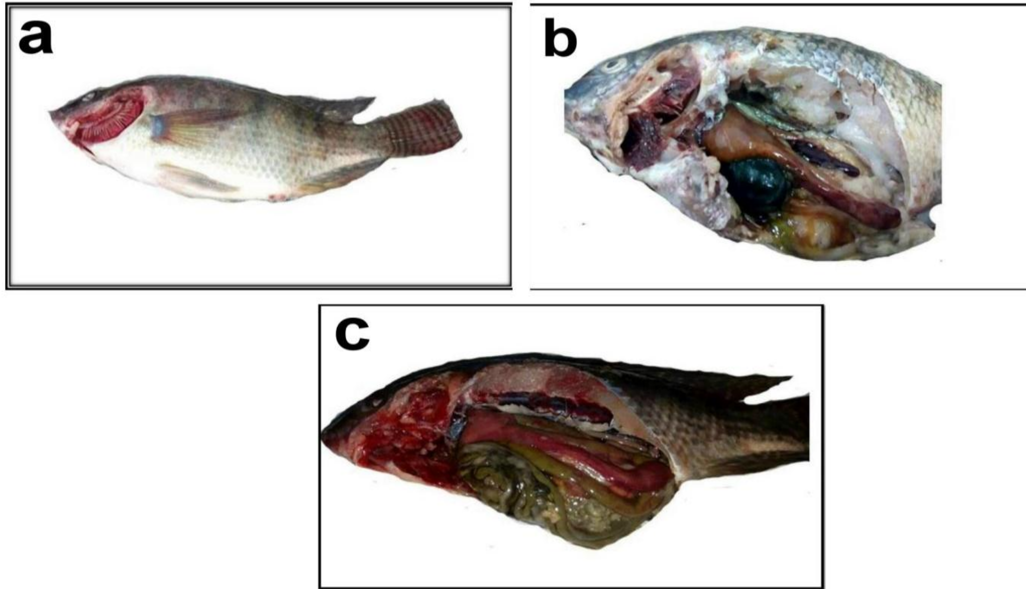

Fig.1a: Naturally infected fish showing erosion of gills.
Fig.1b: Naturally infected fish showing enlargement and congestion of liver and spleen.
Fig.1c: Naturally infected fish showing congestion of kidney.

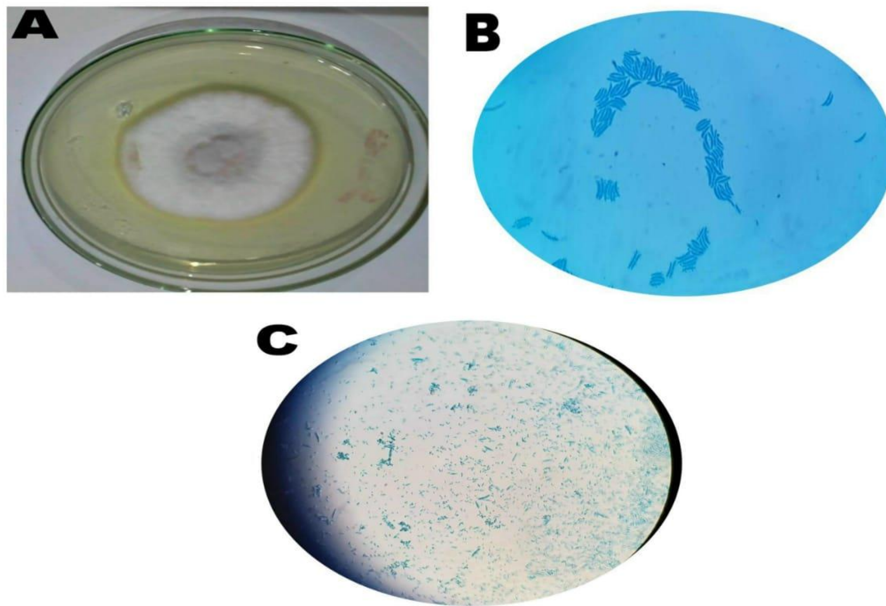

Fig 2a: showing colonies of *fusarium oxysporum* on sabaroud dextrose agar of white mauve mycelium with dense flaccose.
Fig.2b: showing macroconidia of *fusarium oxysporum* stained with lactophenol cotton blue showing slightly curved with and thin wall(x40)
Fig.2c: showing macroconidia of *fusarium oxysporum* stained with lactophenol cotton blue showing fusiform to kidney shaped singly or in pairs (x 40)

3. **Internal transcribed spacer (ITS) sequencing:** *Fusarium oxysporum* was identified based on molecular identification. In the PCR assay, DNA samples extracted from the fungal samples gave the expected fragment sizes of 600 bp for the ITS rDNA regions (figure 3).The fungal isolates were subjected to ITS rDNA sequence analysis and identified as *Fusarium oxysporum*. The sequencing result of the amplified products obtained in the present study has been deposited in the GenBank database under accession number (GenBank MT507863).

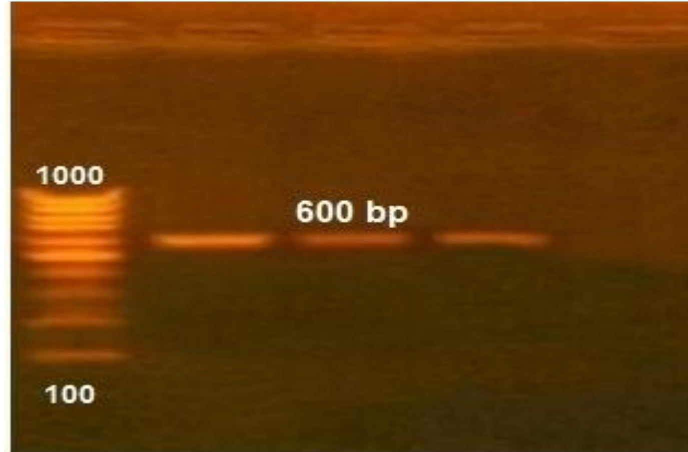

Fig.3: Electrophoresis image from amplification of ITS rDNA of Fusarium oxysporum.

4. **Histopathological alterations demonstrated in *O.niloticus* naturally infected with *Fusarium oxysporum*:**
Severe and variable lesions were demonstrated in the gills, liver and spleen of *O.niloticus* naturally infected with *Fusarium oxysporum*. Lesions in the gills were varied from severe congestion of the central venous sinuses of the primary gill lamellae as well as the blood vessels of the secondary lamellae associated with lamellar edema (fig 4a & 1b), to massive necrosis of the lamellar epithelium (fig 4c), with presence of septated fungal hyphaea intermingled with the disquamated necrotic epithelium (fig 4d). The septated fungal hyphae are also demonstrated in the blood vessels of both the primary gill filament (**Fig.** 4e) and the branchial arch (**Fig.** 4f). In some examined cases, fungal mycetoma are clearly demonstrated in the secondary lamellae (**Fig**. 4g). The gill filaments, in other examined cases, appeared completely denuded of epithelium with the presence of the cartilaginous support only (**Fig.** 4h). Liver showed congestion of hepatoportal blood vessels and sinusoids associated with perivascular edema and intense aggregation of eosinophilic granular cells (EGCs) (fig 5a). Intense perivascular and focal liver hemorrhage were frequently demonstrated in this group (**Fig.** 5b) in addition to massive hepatocellular necrosis, in which the necrotic hepatocytes appeared intensely eosinophilic with pyknotic nuclei concurrently with presence of apoptotic bodies (**Fig.** 5c). The hepatopancrease of all examined cases revealed necrosis of pancreatic acinar cells with intense infiltration of melanomacrophage cells and EGCs (Figs. 5d & 5e). Spleen showed activation of melanomacrophage centers (**Fig.** 5f) and presence ofvariable granulomas embedded in the splenic parenchyma. Each granuloma is consisted of focal aggregation of macrophages and epithelioid cells surrounded by delicate fibrous capsule together with activation of melanomacrophage cells (Fig. 5g).

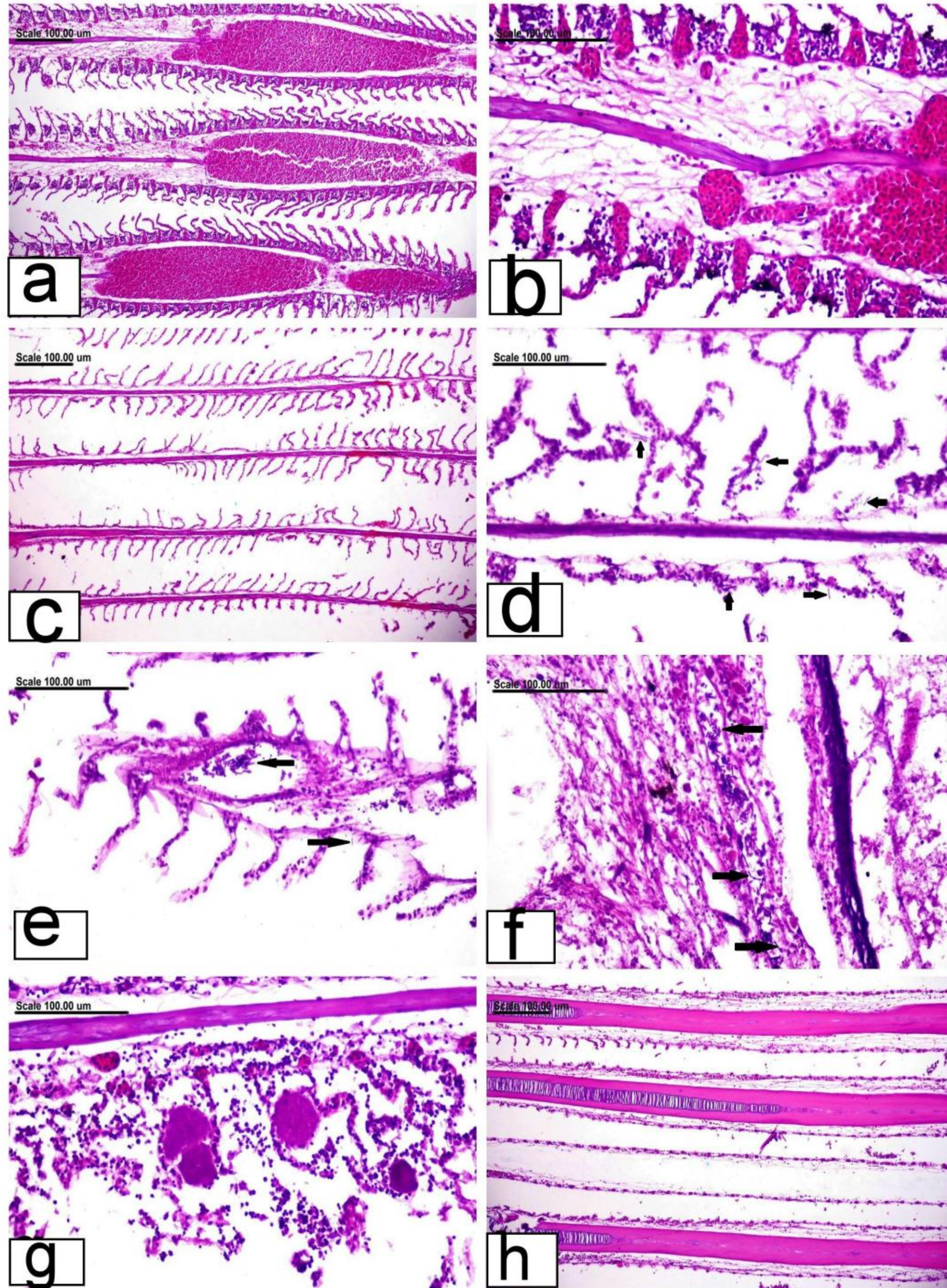

Figure 4: Micrograph of gills of *O.niloticus* naturally infected with Fusarium oxysporum showing, (a,b) congestion of the central venous sinuses of the primary gill lamellae as well as the blood vessels of the secondary lamellae (a) associated with lamellar edema (b), (c) necrosis of the lamellar epithelium, (d, e, f) presence of septated fungal hyphaea intermingled with the desquamated necrotic epithelium (d), and in the blood vessels of both the primary gill filament (e) and the branchial arch (f) (arrows), (g) fungal mycetoma in the secondary lamellae, and (h) presence of the cartilaginous support of the gill filaments which are completely denuded of lamellar epithelium. (stain: H&E, scale bar=100µm).

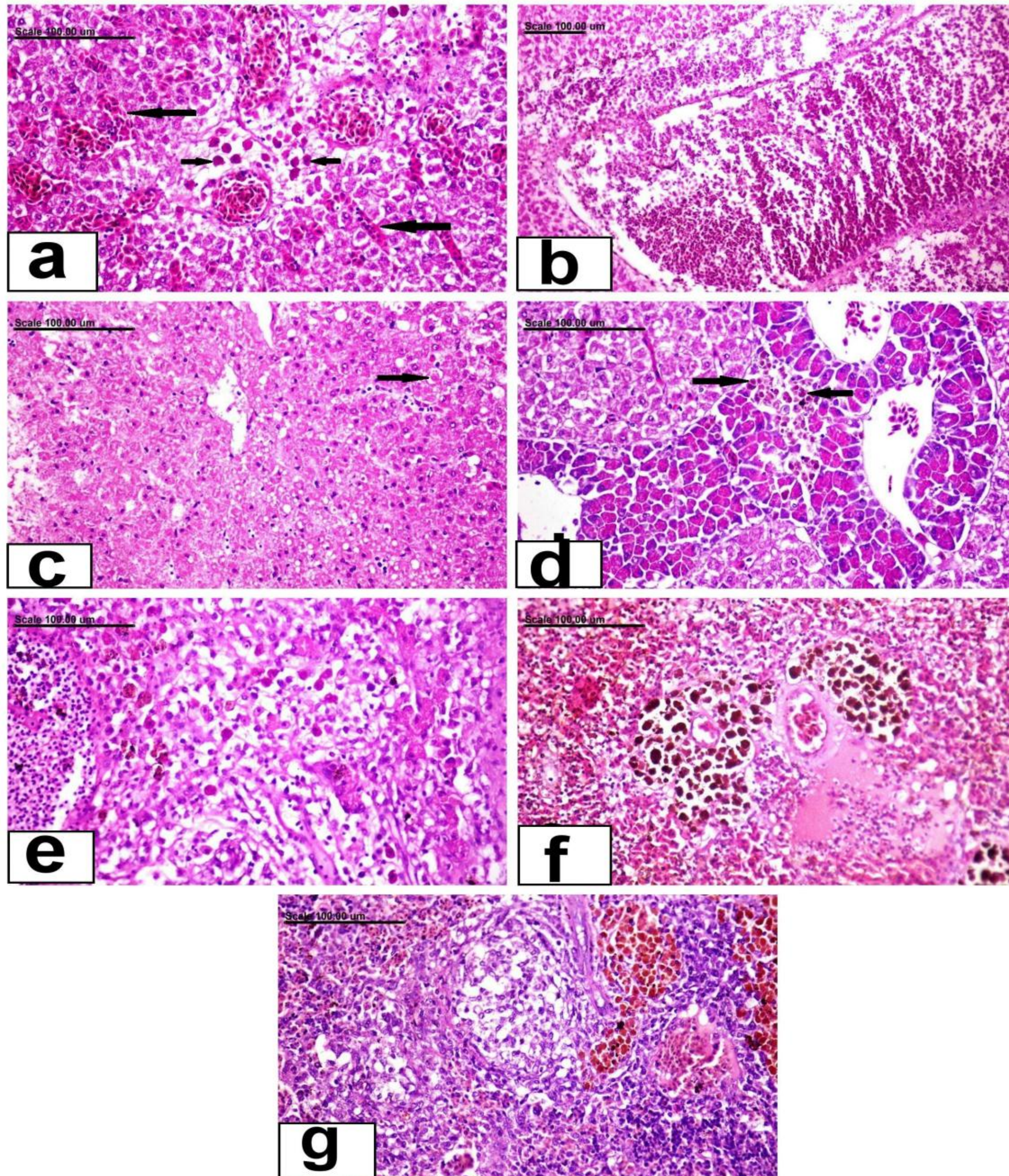

Figure 5: Micrograph of liver and spleen of *O.niloticus* naturally infected with *Fusarium oxysporum* showing, (a) congestion of hepatoportal blood vessels and sinusoids (long arrows) associated with perivascular edema and intense aggregation of eosinophilic granular cells (short arrows), (b) perivascular hemorrhage, (c) massive hepatocellular necrosis concurrently with presence of apoptotic bodies (arrow), (d) infiltration of hepatopancrease with melanomacrophage cells (arrows) and (e) necrosis of pancreatic acinar cells with intense infiltration of melanomacrophage cells and EGCs, (f) Spleen showed activation of melanomacrophage centers and (g) focal granuloma which is consisted of focal aggregation of macrophages and epithelioid cells surrounded by delicate fibrous capsule together with activation of melanomacrophage cells. (stain: H&E, scale bar=100µm).

5. **Experimental infection with fusarium oxysporum and treatment:** the inoculated *Oreochromis niloticus* fish samples started to die on the 4th day after inoculation. Fish became darker and lethargic, with abnormal swimming behaviour, erosion of fins ,ulcer in back region, scattered granulomas were present in visceral organs like kidney and spleen ,exophthalmia ,congestion of liver and enlargement of spleen **(Fig. 6)** . The cumulative mortality in infected group without treatment was 70%. For treated groups with curcumin or H_2o_2, there was the same clinical lesions in mild picture with cumulative mortality 60% and 55 % in both H_2o_2 and curcumin treated groups respectively.

- **Histopathological alterations demonstrated in the tissues of O.niloticus experimentally infected with Fusarium oxysporum and the other treated groups:**
  In general, more pronounced and characteristic histopathological lesions were demonstrated in the different tissues of O.niloticus. These lesions were mainly granulomatous and demonstrated in all organs including the muscle. Muscle of normal control O.nilotocus showed normal muscle fibers with no evidence of necrotic or inflammatory reaction (Fig 7a). But, extensive necrosis and fragmentation of muscle fibers, which are intensely infiltrated with mononuclear cells intermingled with numerous septated fungal hyphae, were demonstrated in the muscles of O.nilotocus inoculated with Fusarium oxysporum (Fig 7b). Additionally, multiple granulomata consisting of central necrotic tissues surrounded by proliferation macrophages and epitheioid cells were also demonstrated (Fig 7c). The fungal hyphae are septate sporodochial conidia, tapering toward both ends with a pointed apical cell and a slightly hooked (Fig 7d). Similar histopathological alterations were recorded in the muscle of curcumin and H2O2 treated fish (fig 7e & 7f, respectively).

- **Fungal isolation:**
  To confirm that *fusarium oxysporum* was responsible for the pathogenicity, the fungus was reisolated from experimentally infected fish on SDA from all infected organs. The results showed the ability of the fungus to infect gills, liver, kidney, spleen . There was no growth on plates for control samples.

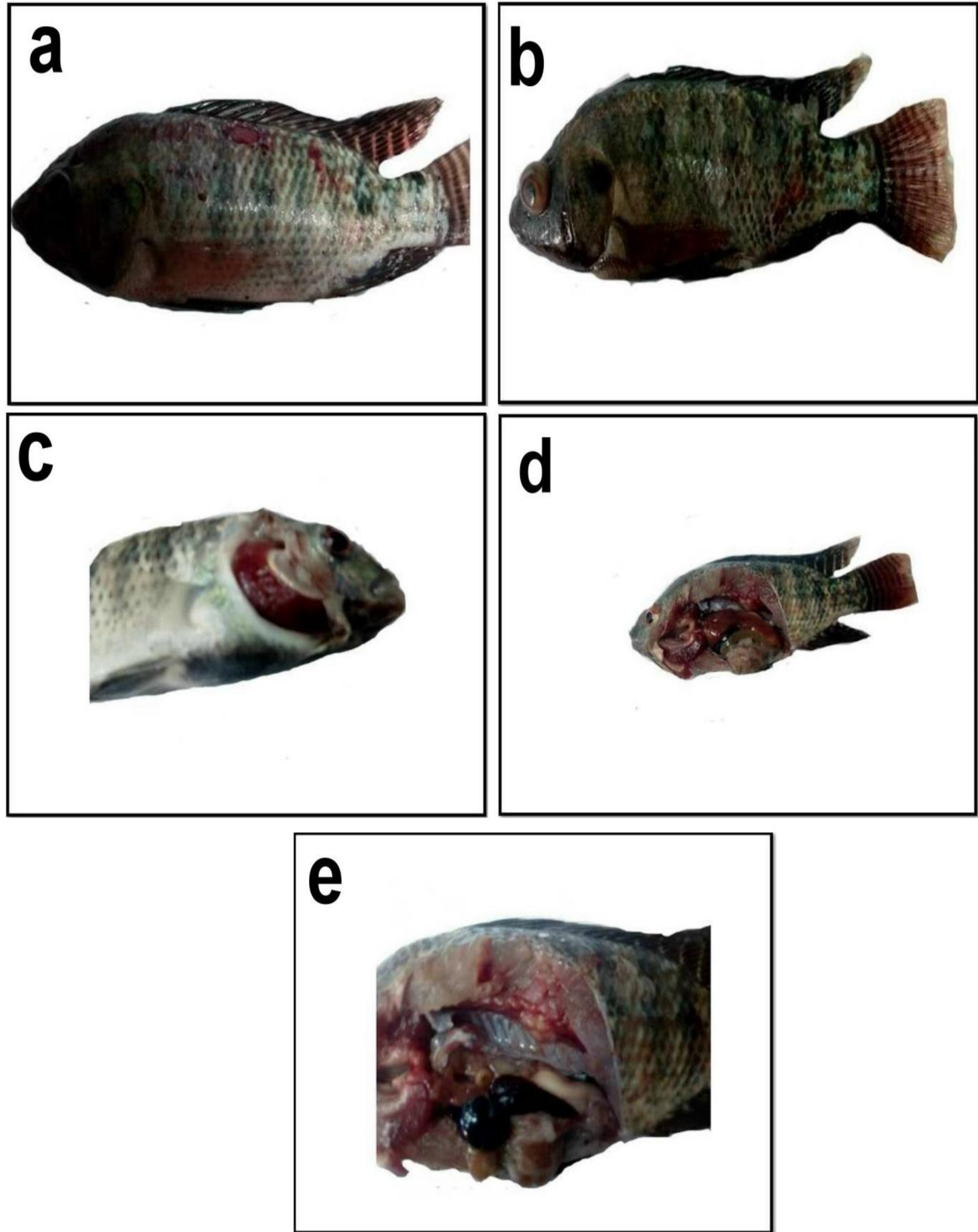

Fig. 6, a: Fish showing ulceration of skin.
Fig.6, b: Fish showing exophthalmia
Fig. 6, c: Fish showing granuloma in gills
Fig. 6, d: Fish showing granuloma in kidney and congestion of liver.
Fig. 6, e: Fish showing enlargement of spleen with scattered granuloma

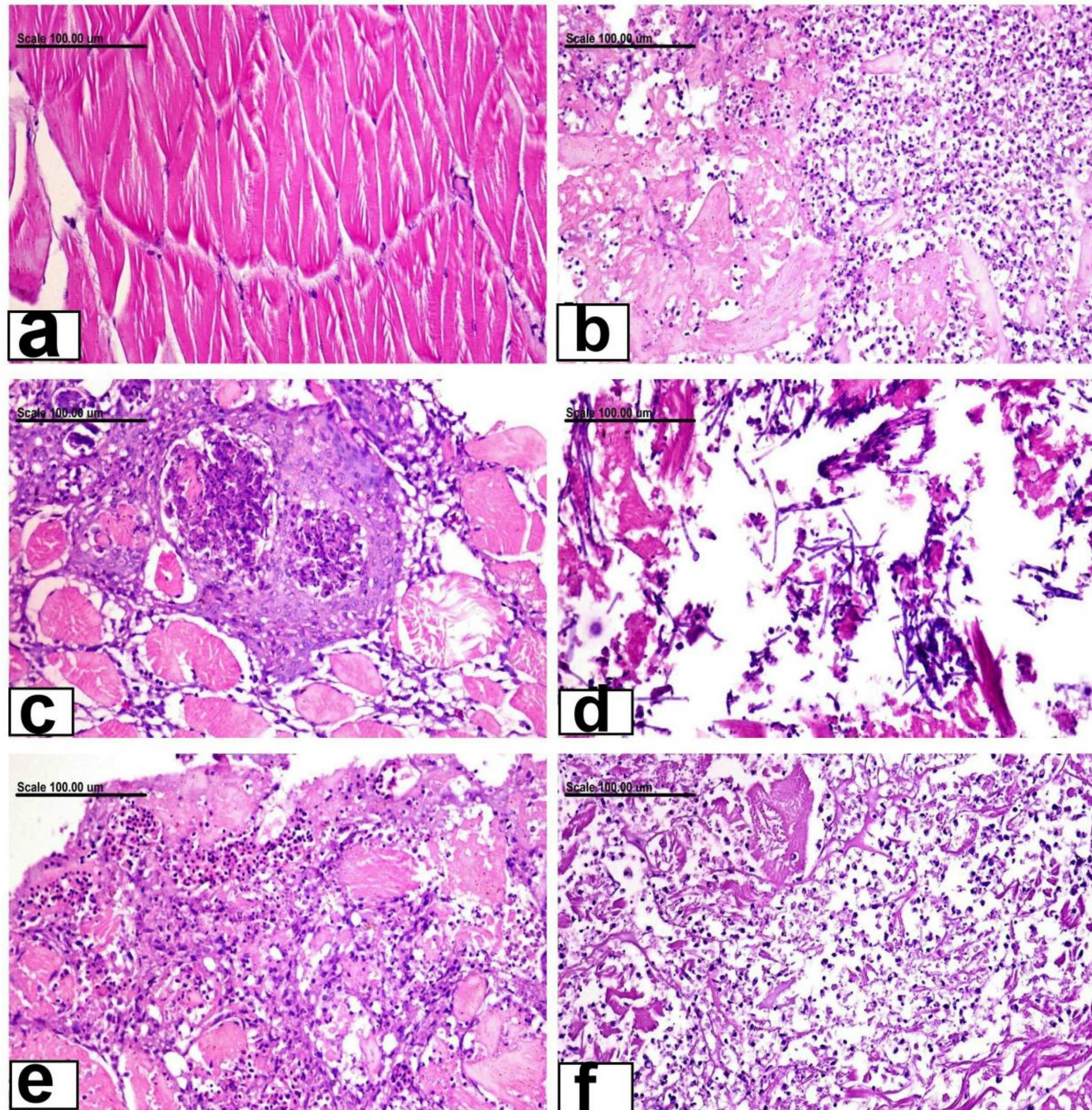

Figure 7: Micrograph of muscle of *O.niloticus*, of (a) normal control *O.nilotocus* showing normal muscle fibers with no evidence of necrotic or inflammatory reaction, (b,c,d) *O.nilotocus* inoculated with *Fusarium oxysporum* showing extensive necrosis and fragmentation of muscle fibers, which are intensely infiltrated with mononuclear cells intermingled with numerous septated fungal hyphae (b) in addition to granuloma consisting of central necrotic tissues surrounded by proliferation macrophages and epitheioid cells (c) as well as the clumps of fungal hyphae (d), (e) curcumin treated *O.niloticus* showing necrosis and fragmentation of muscle fibers and (f) H2O2 treated *O.niloticus* showing the massive mononuclear cells infiltrating the necrotic muscle fibers. (stain: H&E, scale bar=100µm).

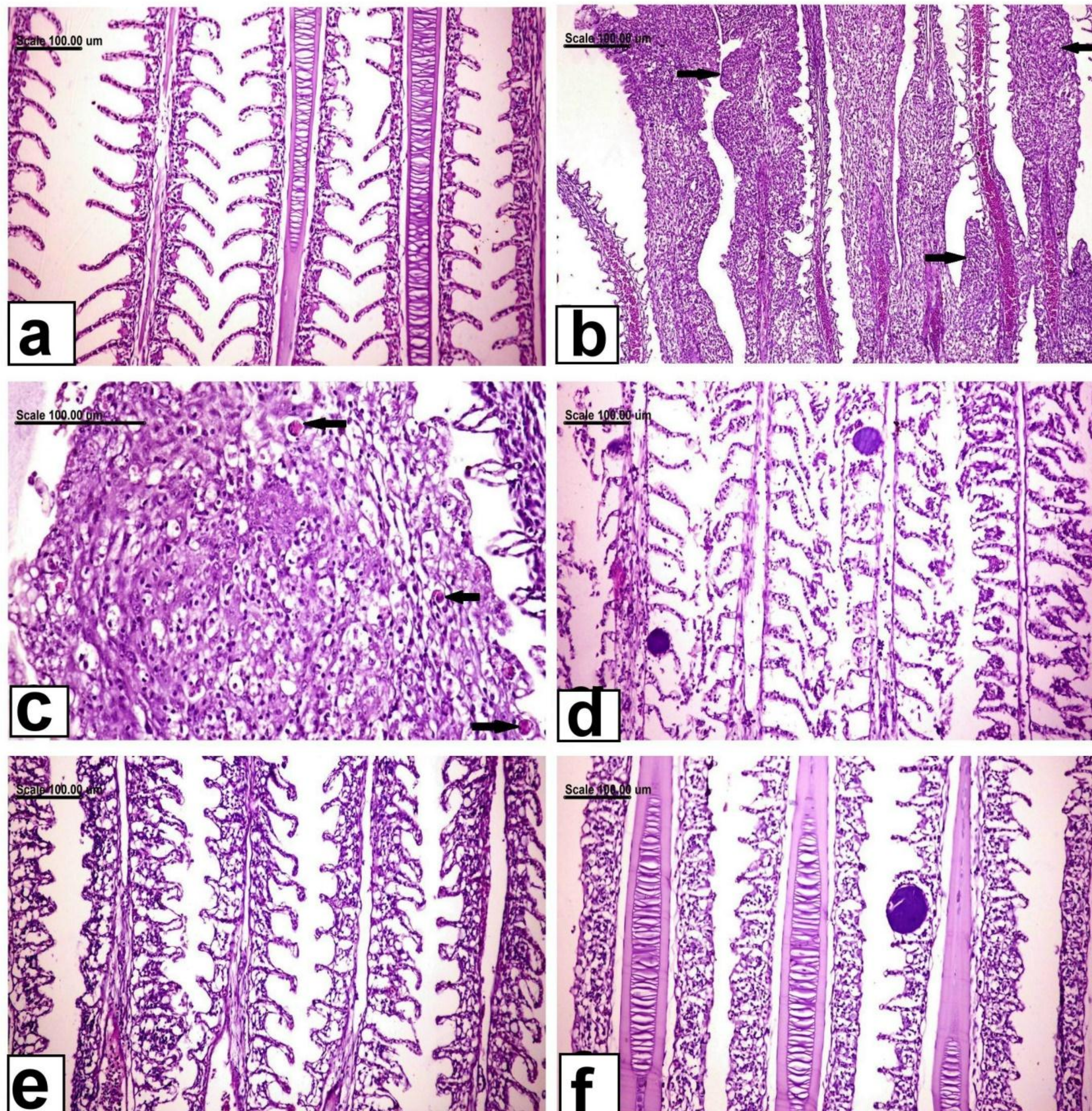

Figure 8: photomicrograph of gills of *O.niloticus*, of (a) normal control *O.nilotocus* showing normal histological structure, (b,c,d) *O.nilotocus* inoculated with *Fusarium oxysporum* showing marked distortion of the gill filaments with diffuse hyperplasia and fusion of gill lamellae in addition to numerous granulomata embedded in the primary and secondary lamellae (arrows) (b), aggregation of macrophages and epithelioid cells associated with abundant apoptotic figures (arrows) (c) and the fungal mycetoma (d), (e) curcumin treated *O.niloticus* showing restoration of gill filaments and (f) H2O2 treated *O.niloticus* showing decreased number of fungal mycetoma. (stain: H&E, scale bar=100µm).

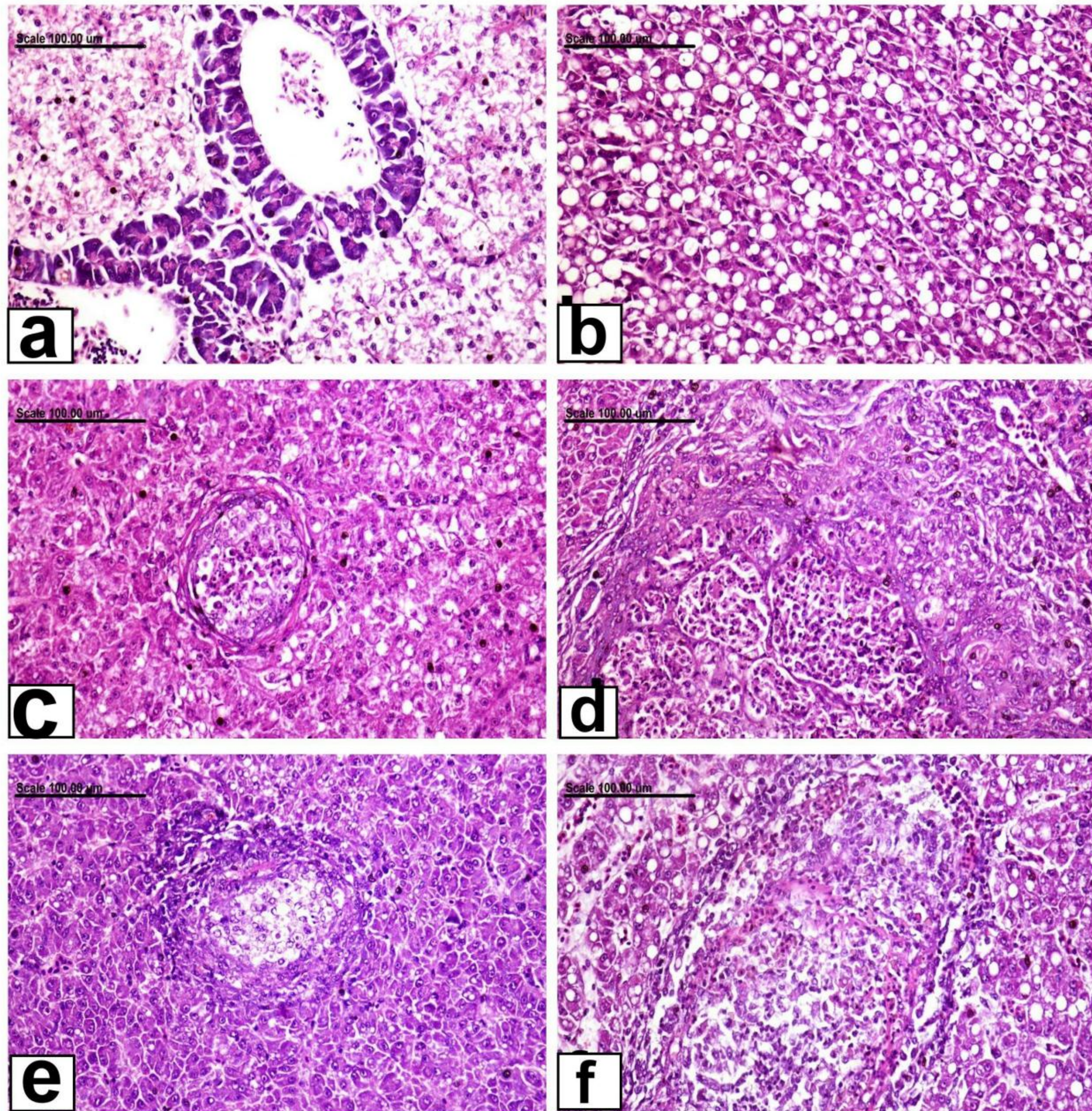

Figure 9: photomicrograph of liver of *O.niloticus*, of (a) normal control *O.nilotocus* showing normal hepatocytes with large vesicular nuclei and normal hepatopancrease, (b,c,d) *O.nilotocus* inoculated with *Fusarium oxysporum* showing diffuse hepatic lipidosis (b), and granuloma, consisting mainly of central eosinophilic necrotic tissue surrounded by macrophages and encapsulated by fibrous tissue, embedded in the hepatic parenchyma (c) and hepatopancrease (d), (e) curcumin treated *O.niloticus* showing small granuloma embedded in the hepatic parenchyma and (f) H2O2 treated *O.niloticus* showing large granuloma in the hepatopancreatic tissue. (stain: H&E, scale bar=100µm).

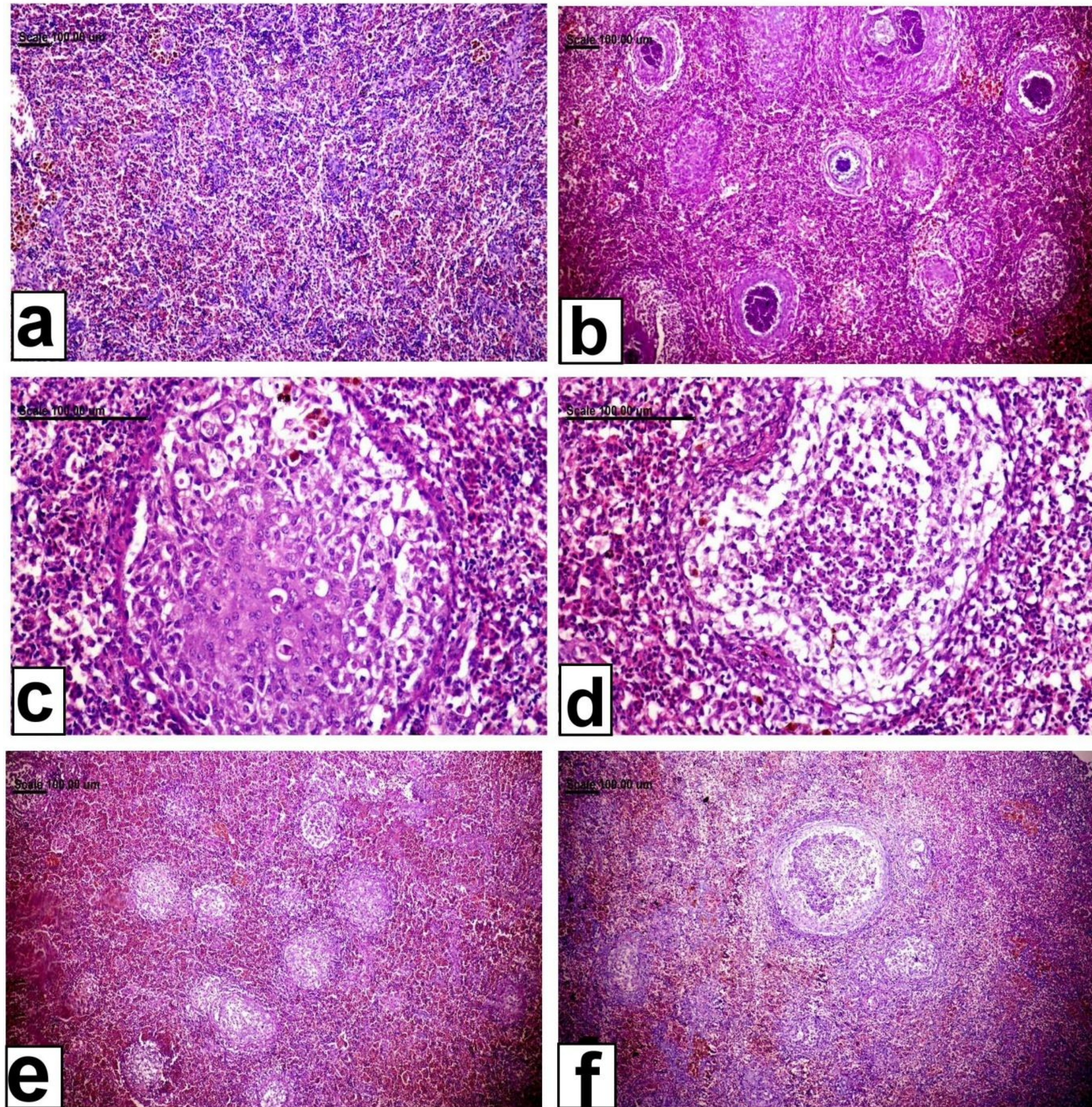

Figure 10: photomicrograph of spleen of *O.niloticus*, of (a) normal control *O.nilotocus* showing normal histological structure, (b,c,d) *O.nilotocus* inoculated with *Fusarium oxysporum* showing numerous granulomas embedded in the splenic parenchyma (b) consisting of aggregation of macrophages, epithelioid cells and melanomacrophage cells surrounded by delicate fibrous stroma (c) and granuloma with eosinophilic fragmented and necrotic center (d), (e) curcumin treated *O.niloticus* showing numerous granuloma and (f) H2O2 treated O.niloticus showing multiple granuloma. (stain: H&E, scale bar=100µm).

DISCUSSION

Fusarium is a large genus belonging to the Ascomycetes containing a few hundred species, distributed primarily in soils and water systems (Palmero,2009). *Fusarium Oxysporum, F.solani* and *F. Verticillioids* have been identified as the most common species that cause invasive fusariosis in humans, especially in patients with immunocompromise (Peterson,2014; Guarro,2013; Latif *et al*.,2012). Fungal infections in fish are usually considered secondary to other pathogens (parasitic diseases or bacteria) or linked to changes in water or the environment that can lead to stress on the animals. However, fungi can cause disease due to different factors, some of which may be more aggressive and play a critical role in infectious diseases (Salter *et al* .,2012; Yanong,2003). Infection with fusarium in fish usually results in dermatitis and systemic lesions and the progression may vary from many days to weeks depending on other factors (e.g. change in water quality or natural sunlight). (Yanong,2003).In the present study, naturally infected fish showing different clinical lesions such as erosion of gills, enlargement and congestion of liver and spleen and congestion of kidney. Histopathological examination of naturally infected *Oreochromis niloticus* revealed different clinical abnormalities of gills, liver and spleen in addition to the appearance of fungal mycetoma and septated hyphae .These results are more or less similar to those of Refai *et al*.(2010) who isolated fusarium from Nile tilapia and cat fish showing similar clinical abnormalities and confirmed lesions through histopathological investigation. Fusarium infection was recorded sporadically in fish and other aquatic species, but identification was often limited to the level of the genus, or based solely on morphological characteristics (salter *et al* .,2012; Abd El-Ghany *et al* .,2014). The recorded clinical signs, mortality rate and histopathologigical changes in Fusarium infected *O.niloticus* could be attributed to the virulence factors of *Fusarium oxysporum*. In this respect, Thatcher *et al*.(2016) reported the virulence factors responsible for the pathogenicity of *F. oxysporum* .Study revealed that the major genes involved in pathogenesis are associated with degradation of proteins and carbohydrates/sugars as well as membrane transport and oxidative processes. Also, it may be due to the number and morphology of conidia injected. Concerning this point, Coleman *et al*. (2011) stated that the pathogenesis of Fusarium sp. is based on the number and morphology of the conidia injected. Microconidia are more virulent than their microconidia counterpart is. However, the exact role of these structures during pathogenesis in fish need further details and investigations in the future.

Fusarium species identification is quiet difficult and requires a specialized laboratory and qualified personnel. In this study isolated Fusarium sp. identified as Fusarium oxysporum based on macroscopical and microscopical examination which compatible with the fungi report from prawn culture systems in Japan (Hatai 2012).Full identification of the isolated Fusarium was done through internal transcribed spacer sequencing which

agree with that of Salighehzadeh *et al*.(2019). The experimental infection of F. oxysporum into kuruma prawn (*Penaeus japonicus*) and Nile tilapia (O. niloticus) was successfully developed and pathogenicity of F. oxysporum was confirmed on aquatic animals (Khoa & Hatai 2005; Cutuli *et al*. 2015). Development of the infection during this pathogenicity test and histopathologic findings indicated the virulence of the fungi and potential risk of spreading among other healthy fish communities. This study may be the first trial for treatment of Fusarium *oxysporum* infection in cultured *Oreochromis niloticus* using curcumin as antifungal treatment in aquaculture. Curcumin in this study had mild effect as antifungal against *fusarium oxysporum* in Nile tilapia. This result disagrees with that of Liu *et al*.(2017)who showed that curcumin can be used as a potential compound for the development of commercial drugs against I. multifiliis. In this study hydrogen peroxide showed mild effect against *fusarium oxysporum* infection in *Oreochromis niloticus,* This study disagree with that of Novakov *et al*.(2018)who brought greatest effect against saprolegniosis in Brown Trout (*Salmo trutta*) Eggs. Otherwise, this result agree with that of Abd El Aziz *et al*.(2004)who mentioned that hydrogen peroxide had mild effect against saprolegniosis on large scale of Nile tilapia in fish farms. Further investigations are needed to determine the optimum concentration of both curcumin and hydrogen peroxide in treatment of fusariosis in freshwater fishes.

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