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DIFFERENTIAL EXPRESSION OF SECRETED ASPARTYL PROTEASE (SAP8 AND SAP10) GENES AND COMPARATIVE PATHOGENICITY OF *Candida albicans* GROWN IN VARIOUS ENVIRONMENTAL CONDITIONS

Inas K. Rahem[*],Hayder M. Samaka[*] , Hutheyfa A. Al Salih[**]

[*]Department of Microbiology, Faculty of Veterinary Medicine,University of Kufa,Kufa,Iraq.

[**]Department of Pathology, Faculty of Veterinary Medicine,University of Kufa,kufa,Iraq

Corresponding Author: enase503@gmail.com

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ABSTRACT

This study focusing on the importance of *Candida albicans* growth conditions on SAP8 and SAP10 genes expression, as a member of the Secreted Aspartic Proteases superfamily genes that play a role in the pathogenicity of *C. albicans* and the effects of this conditions on the pathogenicity of this bacterium in albino mice.Ten pathogenic isolates of *C. albicans* were grown on two different conditions using RPMI1640 medium at 37° C for mimic host condition and on Sabouraud Dextrose Agar (SDA) at 25° C as in vitro condition. Sets of primers were used to detect SAP8 and SAP10 genes expression in each condition. Forty BALB/c albino mice were assigned to groups and inoculated with 1 X 10^7 cells/mouse of *C. albicans* grown in the study conditions as challenge dose. Kidneys, lungs, and liver were collected to study the pathological changes. Data show overexpression of the SAP8 gene in study isolates grown in RPMI1640 comparison to the SAP10 gene. Kidneys, liver, and lungs showed pathological lesions at a different range of severity, a significant severe lesion in the kidney in mice injected with *C. albicans* grown in RPMI 1640 medium, while in contrast the significant severe liver and lung lesions were observed in mice injected with *C. albicans* grown in SDA medium. This study points out that the growth condition of *C. albicans* plays a role in the pathogenicity of this microorganism and SAP8 gene related to the infection process in the host.

INTRODUCTION

Candida albicans is polymorphic fungus considered as a member of the normal microflora in human and animals and exist as a lifelong, harmless microbiome in varied hosts, but in some conditions, related to the host or environment, *C. albicans* can become a serious pathogen, leading to a variety of infections range from superficial infections of the skin to fatal systemic infections (1, 2).

The ability of *C. albicans* to cause infections is supported by a group of factors and attributes which considered as virulence factors, including the morphological transition between yeast and hyphal forms (phenotypic switching), adhesions and invasions, biofilms formation, and secretion of hydrolytic enzymes (3-6).

Secreted aspartyl proteinases, phospholipase B, and lipases are the most important exoenzymes produced from *C. albicans* and play an important role in the pathogenicity of this microorganism (7), Secreted aspartyl proteinases (SAPs) are enzymes encoded by a group of superfamily genes (SAP1 to SAP10), most of them has a defined role and action in the pathogenicity of *C. albicans* in adhesion, invasion and tissue destruction (6 - 8), but still, the full functional repertoire of the Sap genes family has yet to be revealed(7).

(9) revealed a correlation between SAP8 expression and biofilm formation. Also, in pathogenic fungi, mitogen activated protein kinase (MAPK) pathways regulate dimorphism, biofilm/mat formation, and virulence. The reduction in SAP8 expression inhibit MAPK activity led to fail in biofilm formation (10).

While SAP10 have a roles in cell surface integrity and adhesion (11-14), while *Candida albicans* showed a variation in its abilities to adhesion and biofilm formation, when cultured in different growth conditions (15).

The present study was conducted to investigate the levels of SAP8 and SAP10 genes expression in *Candida albicans* that grown in two different conditions, RPMI1640 (to mimicking host conditions *in vitro*) and Sabouraud dextrose agar (SAD) as laboratory conditions, also to

investigate the pathological features of *Candida albicans* that grown in these two conditions in albino mice.

MATERIAL AND METHODS

Secreted Aspartyl Proteinase 8 and 10 gene expression analysis

Ten pathogenic isolates of *Candida albicans*, obtained from clinical cases in previous study [16], were included in this study, the isolates were cultivated on Sabouraud dextrose agar (SDA) nightlong at 25^{o}C and then 0.5 ml of stocks suspension (OD_{600}=0.1) from each isolates were cultivated on two different conditions: laboratory conditions (on SDA at 25°C) and host conditions (on RPMI1640 at 37°C), after 12 hours; all isolates were harvested and prepared for RNA extracted using YeaStar RNA Kit from (ZYMO Research, USA).

Set of primers from (17,18) were used for analyze SAP8 and SAP10 gene expression using ACT1 gene as internal control (Table 1). The relative change of SAP 8 and SAP10 gene expression was done by comparing the genes expression levels on the RPMI1640 medium as a target to the genes expressions on SDA medium as a calibrator using (ACT1) gene as a reference gene (19), data analyzed by MxPro qPCR software from (Agilent, USA).

The following protocol was used in the real-time PCR system (MX 3005P system, Agilent, USA) using One step EvaGreen qRT-PCR kit (Applied Biological Materials Inc., USA): one cycle on 42°C for 15 min., 95°C for 10min. for initial denaturation and 40 cycles as follow: 95°C for 15 sec. , 60°C for 60sec., then melting curve cycle as instrument instruction (50-95°C).

Table 1: Primers set used in real-time PCR for the quantification of the expression of *C. albicans* SAP 8 and SAP 10 genes.

Primer	Primer sequence		Reference
ACT1	R	5'-ATGGGATGAATCATCAAACAAGAG-3'	(18)
	F	5'-TTTCATCTTCTGTATCAGAGGAACTTATTT-3'	
SAP8	R	5'-GGTGTTCCCATCAAGATCATAAACT-3'	(19)
	F	5'-GGTGTTAGTAGAGATCTGGCCACTATT-3'	
SAP 10	R	5'-CCGTCCTTTTCAGTCTTGAGATC-3'	
	F	5'-GGTTTTCGATAGGCGATTGAGA-3'	

Experimental infections

To investigate the pathogenicity of *C. albicans* in vivo, all experimental animals were inoculated by study isolates grown on two different study conditions at dose of $1X10^7$ cells/mouse intravenously (tail vein) to induce sub-acute systemic infection (20). Forty BALB/c albino mice (bodyweight range 20 to 30 gm) were assigned to five groups (eight mice each group) namely, G1, G2, G3 and G4, in addition to GC group as control group mice were received one injection of normal saline at day one of the experimental period. Group G1 and G2 mice were inoculated with *C. albicans* isolates grown in (RPMI1640) at day one of experimental period and group G3 and G4 mice were inoculated with isolates grown in (on SDA medium) at day one of experimental period. Mice were euthanized after 30 days of experimental period by exsanguination under general anesthesia, the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and Institutional Animal Ethics Committee (IAEC) instructions in the university of Kufa have been followed in the handling of the laboratory animals.

Lesion scoring system

Lesion scoring system was conducted to observe the significance of pathological changes in experimental animals. The lesion scoring system criteria were written down according to observation, monitoring, and assessment the lesion in kidney, lung and liver samples of the whole present study (Table 2). The analysis data obtained, systematic lesions score was designed under the supervision of group of Pathologist in the Faculty of Veterinary Medicine-University of Kufa.Lesion scoring system was divided into four grades normal, mild, moderate and severe with values 0, 1, 2, 3 and 4, respectively. The histopathological lesion scoring results were analyzed statistically using Kruskal Wallis non-parametric one-way ANOVA test while using Mann Whitney *U* test to compare between the groups.

Table 2: Lesion score criteria of lungs, kidneys and liver in the experimental animals, the lesions score was designed under the supervision of group of Pathologist in the Faculty of Veterinary Medicine-University of Kufa.

Lung

Lesion score	Lesion severity	Lesion score description
0	Normal	Normal lung histology
1	Mild	1-Hyperplasia of alveolar walls in the focal area in pulmonary parenchyma, 2-Congestion, 3-Small focal areas of infiltrated inflammatory cells.
2	Moderate	1-Severe congestion of all blood vessels lumen, 2-Observation of moderate hemorrhage in pulmonary parenchyma, 3-Presences of small masses of inflammatory cells and some necrotic debris of pneumocytes, 4-The alveolar walls of certain areas in the lung were thickened due to hyperplasia of pneumocytes.
3	Sever	1-Severe massive hyperplasia of alveoli leads total close of alveolar spaces forming compact tissue feature in contrast to normal lung histology of spongy tissue feature led to the loss of normal pulmonary architecture, 2-Infiltration of inflammatory cells within the hyperplastic cells and also in large masses mixed with necrotic tissue debris, 3-Severe massive hemorrhage in all lung parenchyma.

Kidney:

Lesion score	Lesion severity	Lesion score description
0	Normal	Normal kidney histology
1	Mild	1. Congestion of renal blood vessels, 2. Infiltration of inflammatory cells aggregated in small areas in kidney cortex, 3. Mild necrosis in renal tubules with the presence of pyknosis in epithelial cells of tubules.
2	Moderate	1. Aggregation of inflammatory cells mixed with necrotic debris of renal epithelial cells of proximal and distal convoluted tubules in the cortex or in the loop of Henle tubules in medulla forming a masses parenchyma of kidney, 2. Absence of renal tubules structure of cortex due to necrosis of epithelial cells in focal areas, 3. Severe congestion almost in all renal blood vessels. 4. Hemorrhage also observed in the renal parenchyma, 5. Congestion of glomerulus of capillaries loop.
3	Sever	1. Massive necrosis of epithelial cells of proximal and distal convoluted tubules with the presence of inflammatory cells within necrotic tissue led to loss of normal cortex architecture, 2. Necrosis of loop of Henley tubules led, absence of tubules structure in the medulla, 3. Aggregation of inflammatory cells mixed with the debris of necrotic tissue forming a large masses in cortex or medulla of the kidney, 4. Presence of fungus colonies in the medulla area mixed with inflammatory cells (especially neutrophil) in medulla was observed in certain cases, 5. Presence of pus feature lesion of the mixture of inflammatory cells and necrotic tissue forming a large mass in the medulla, 6. Massive hemorrhage observed in all kidney parenchyma due to destruction of blood vessels. 7. Some glomerulus appeared hyperplastic with an absence of capillaries loop forming a consolidated cellular mass inside bowman capsule, 8. In certain cases, severe lesion showed cyst feature lesion manifested by the formation of huge cysts lined by very thin cells with the presence of the lesions, which described above.

Liver:

Lesion score	Lesion severity	Lesion score description
0	Normal	Normal liver histology
1	Mild	1. Infiltration of inflammatory cells in limited numbers aggregated near portal areas, 2. Congestion of blood vessels of portal areas, 3. Mild necrosis signs observed presented by hepatocytes pyknosis.
2	Moderate	1. Necrosis of hepatocytes was observed near portal area with the presence of fatty liver changes in certain liver lobules, 2. Aggregation of inflammatory cells in clusters within liver parenchyma and some of these clusters contain necrotic tissue debris. 3. Hemorrhage observed in the area surrounded the portal areas (not in centric to lobules), 4. Congestion also observed in most hepatic blood vessels.
3	Sever	1. Massive necrosis of hepatocytes with severe fatty liver changes were observed in all lobules of liver sample and this change manifested by presence of triglyceride molecules forming a large vacuole in the cytoplasm, even larger than the original cells size in some affected cells, 2. Disappearance of hepatocytes chain structure with the absence of hepatic sinusoid due to the massive necrosis of hepatocytes either sinusoid endothelial cells, 3. Massive hemorrhage was spread in all liver parenchyma due to destruction of central veins, endothelial sinusoid cells necrosis or portal area blood vessels, 4. Presence of inflammatory in all affected areas of liver parenchyma within the necrotic tissues or aggregated as clusters, 5. Loss of normal liver architecture

RESULTS

Sap8 and SAP10 gene expression analysis:

This study showing that the SAP8 and SAP10 gene expression levels were different in different *Candida albicans* isolates, and SAP8 gene expression levels were significantly higher than SAP10 gene expression in *C. albicans* that grown in PRMI1640 medium compared with *C. albicans* grown in Sabouraud dextrose agar, while study isolates showed non-significant differences between SAP8 and SAP10 gene expression in *C. albicans* that cultured in RPMI1640 medium (Figure 1).(Table 3)

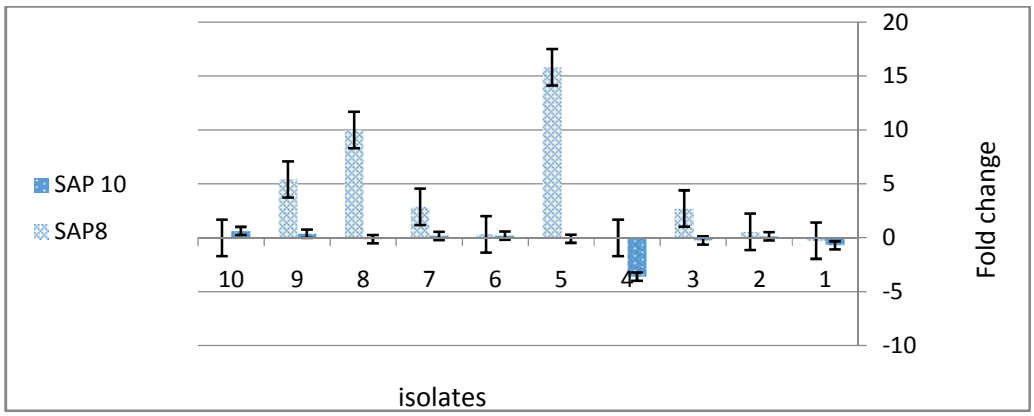

Figure 1: qRT-PCR fold change of SAP8 gene and SAP10 gene in *Candia albicans* isolates growing in RPMI 1640 medium comparing to *Candia albicans* isolates growing in SAD medium.

Table 3: Relative expression of (SAP8 and SAP10) genes in *Candida albicans* isolates grown in RPMI1640 medium compared to the SDA medium as a reference.

Type of gene	Gene expression (in RPMI 1640 medium as target)	Gene expression (in SAD medium as control)	T test	P value
	means± SE	means± SE		
Sap 8	3.748±1.68[a]	0±0[b]	2.325	0.047*
Sap 10	-0.316±0.38 [a]	0±0 [a]	0.831	0.428**

* Significant difference (P <0.05), ** No significant difference (P >0.05)

Histopathology

Kidneys, Liver and lungs samples of mice treated with *Candida albicans* showed a pathological lesion at a different range of severity compared with control mice.In the kidney, the lesion was observed at a range of severity from normal to severe grade, however severe lesion was observed in 50% of groups G1 and G2 mice (Table 4).

Table 4: Lesion severity distribution of kidney of groups G1, G2, G3 and G4 mice.

Group	**Lesion grade**				**Total mice no.**
	Normal	**Mild**	**Moderate**	**Severe**	
G1	1 (12.5%)	1 (12.5%)	2 (25%)	4 (50%)	8
G2	Nil	3 (37.5%)	1 (12.5%)	4 (50%)	8
G3	4 (50%)	3 (37.5%)	1 (12.5%)	nil	8
G4	4 (50%)	4 (50%)	nil	nil	8

The lesion of kidney was characterized by total absence of renal tubules features of cortex and medulla areas, also cystic lesion was manifested in cyst lined by one layer of cuboidal epithelial cells with empty spaces was observed (Fig 2).

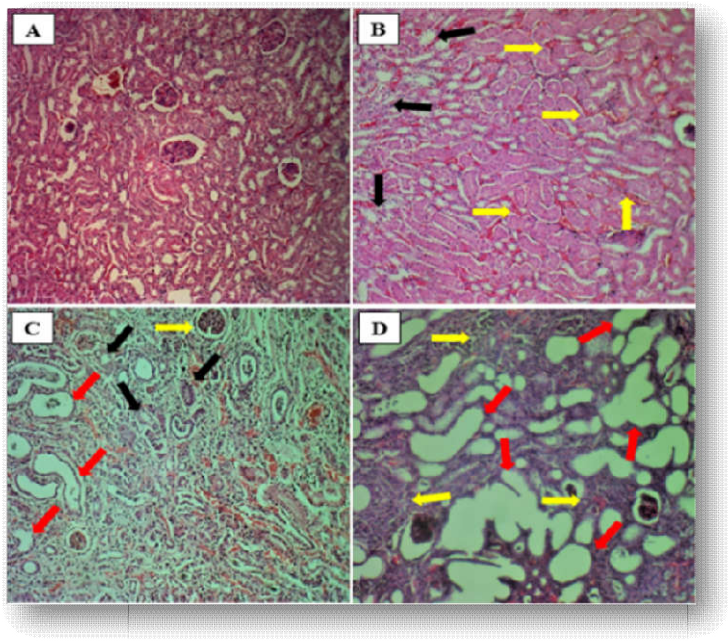

Figure 2 : Photomicrographs of kidney. A/ Normal histology of kidney. B/ Mild grade lesion of kidney. Mild grade lesion was characterized by presence of hemorrhage (yellow arrows) within renal tubules of cortex and medulla and destruction of limited numbers of loop of Henley tubules (black arrows) in kidney medulla was observed. C/ Moderate grade lesion of kidney. The moderate grade lesion was manifested in destruction of renal tubules in cortex area with inflammatory cells that infiltrated in affected area, note absence the most of proximal or distal convoluted tubules feature that surrounding the glomerulus (yellow arrow) with presence of renal tubules individuals (black arrows) in affected area led to loose of kidney architecture in affected area, also few numbers of cystic lesion of kidney (red arrows) was observed. Also, hemorrhage was observed in affected area. D/ Severe grade lesion. The severe grade lesion was characterized by total absence of renal tubules features that replaced by large clusters (yellow arrows) of inflammatory cells formed with presence of severe cystic lesion (red arrows) within this clusters in cortex area led to loose of kidney normal architecture completely. H&E. A, B, C and D: 100x.

The fungus was colonized in medulla areas with presence of inflammatory cells within the fungi colonies, however fungi colonies were observed in groups G1 and G2 mice and did not observe in groups G3 and G4 mice (Fig. 3).

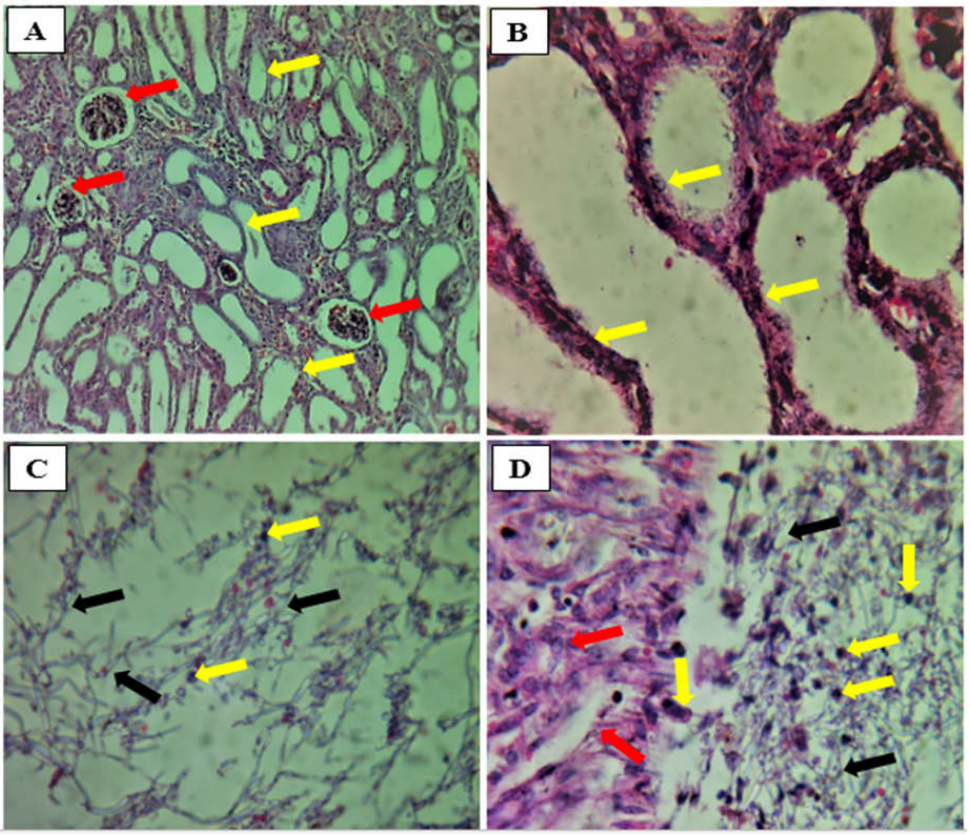

Figure 3 : Photomicrographs of kidney. A/ The severe grade lesion was characterized by presence of cyst features (yellow arrows) extended over all cortex, surrounded by inflammatory cells. Note the total absence of all renal tubules structure, where the glomerulus (red arrows) were surrounded with renal cysts. B/ Renal cysts lesion was vary in size with empty spaces, and this cysts were lined by one layer of cuboidal epithelial cells (yellow arrows). C&D/ *Candida albicans* colonized in medulla area of kidney. Note the *Candida albicans* hyphae (black arrows) mixed with inflammatory cells (yellow arrows), and closed to loop of Henley tubules (red arrows). H&E. A: 100x, B, C and D: 400x.

In the liver, the lesion was observed at a range of severity from normal to severe grade (Table 5), significantly severe lesion was observed in 87.5% of group G4 mice.

Table 5: Lesion severity distribution of liver of groups G1, G2, G3 and G4 mice.

Group	Lesion grade				Total mice no.
	Normal	Mild	Moderate	Severe	
G1	3 (37.5%)	1 (12.5%)	3 (37.5%)	1 (12.5%)	8
G2	4 (50%)	3 (37.5%)	1 (12.5%)	nil	8
G3	1 (12.5%)	3 (37.5%)	4 (50%)	nil	8
G4	nil	nil	1 (12.5%)	7 (87.5%)	8

The lesion of liver was characterized by diffuse severe fatty liver degeneration in all liver parenchyma with severe necrosis of hepatocytes forming spaces in parenchyma led to loose of liver normal architecture (Fig 4&5).

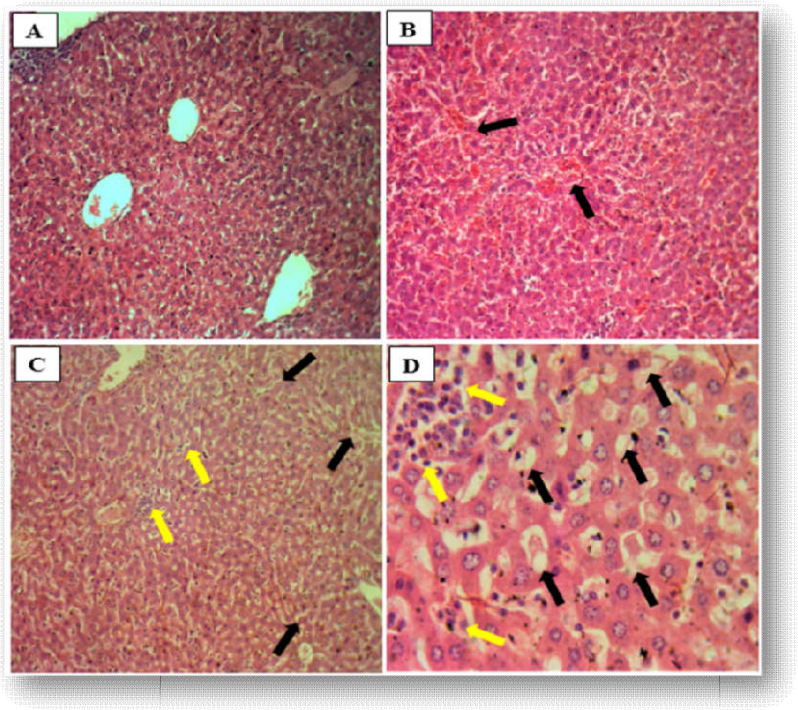

Figure 4: Photomicrographs of liver. A/ Normal histology of liver. B/ Mild grade lesion of liver. The mild grade lesion was manifested in presence of hemorrhage (black arrows) in liver parenchyma, also psychosis or Karyorrhexis of hepatocytes were observed. C&D/ Moderate grade lesion of liver. Moderate grade lesion was characterized by fatty degeneration (black arrows) of hepatocytes forming a vacuoles in liver parenchyma with presence of inflammatory cells (yellow arrows) aggregated focally near blood vessels with

limit number of these cells were spread in hepatic sinusoid. H&E. A, B and C: 100x, D: 400x.

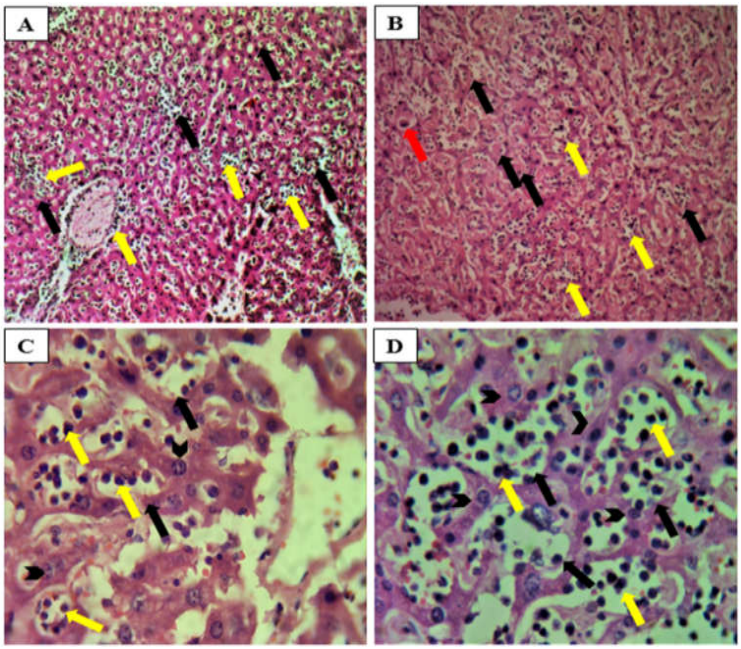

Figure 5 : Photomicrographs of liver. A, B, C&D/ Severe grade lesion of liver. Severe grade lesion was characterized by observation of severe fatty liver degeneration in all liver parenchyma with severe necrosis of hepatocytes forming a spaces (black arrows) in parenchyma led to loose of liver normal architecture, note the individuals of hepatocytes between the necrosis spaces (Arrow heads). Inflammatory cells (yellow arrows) spread in the spaces of necrotic hepatocytes and near blood vessels of the affected areas. Also, giant cells (red arrow) was observed in affected area with presence of congestion in blood vessels. H&E. A&B: 100x, C&D: 400x

In the lungs, the lesion was observed at range of severity from normal to severe grade (Table 6). However, normal lung histology did not observed in groups G3 and G4 mice and lesion was between mild to moderate grades.

Table 6: Lesion severity distribution of lungs of groups G1, G2, G3 and G4 mice.

Group	Lesion grade				Total mice no.
	Normal	Mild	Moderate	Severe	
G1	6 (75%)	1 (12.5%)	1 (12.5%)	nil	8
G2	1 (12.5%)	6 (75%)	nil	1 (12.5%)	8
G3	nil	4 (50%)	4 (50%)	nil	8
G4	nil	5 (62.5%)	3 (37.5%)	nil	8

The lesion of lung was characterized by massive hyperplasia of pneumocytes with diffuse infiltration of inflammatory cells led to total absent of alveolar features of lung parenchyma and losing the normal lung architecture completely (Fig 6&7).

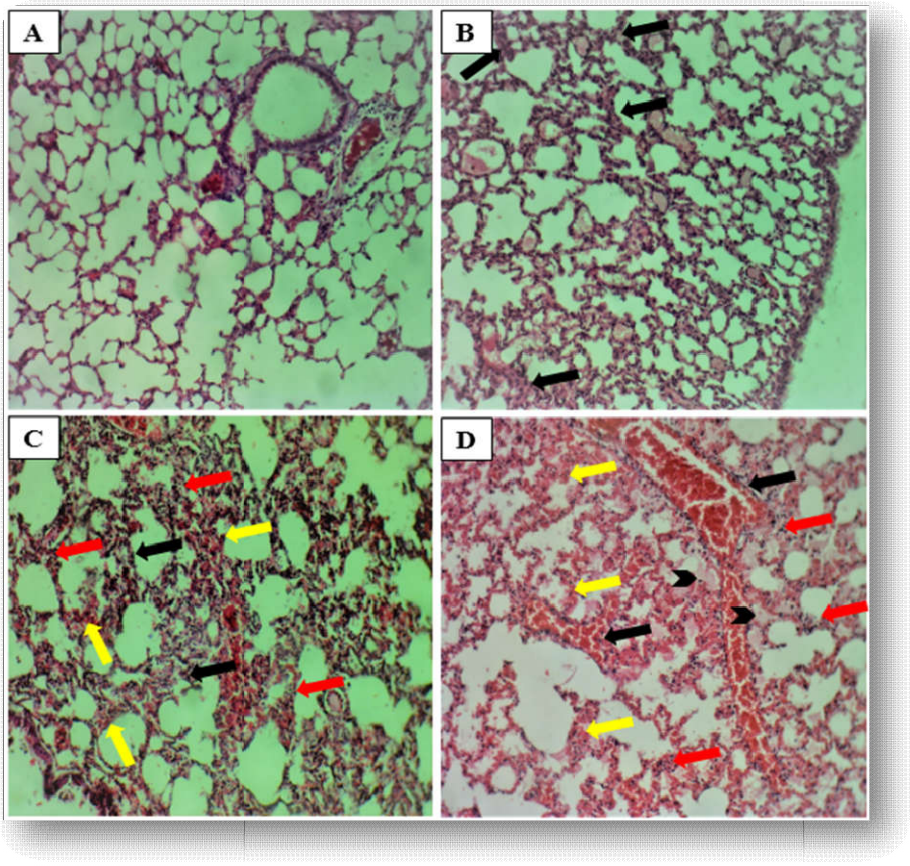

Figure 6: Photomicrographs of lungs. A/ Normal histology of lung. B&C/ Mild grade lesion of lung. The mild grade lesion was characterized by mild thickness of alveolar walls (black arrows) due to congestion of alveolar capillaries (yellow arrows),

hyperplasia of pneumocytes and infiltration of inflammatory cells (red arrows). Blood vessels congestion was also observed. D/ Moderate grade lesion of lungs. Moderate grade lesion was characterized by severe hemorrhage in lung parenchyma with congestion (black arrows) of blood vessels. Thickening of alveolar walls due to alveolar capillaries congestion (yellow arrows), hyperplasia of pneumocytes and infiltration of inflammatory cells (red arrows) was observed. The thickening of alveolar walls led to narrowing the alveolar spaces, which filled transudate fluid (arrow heads). H&E. A, B, C &D: 100x.

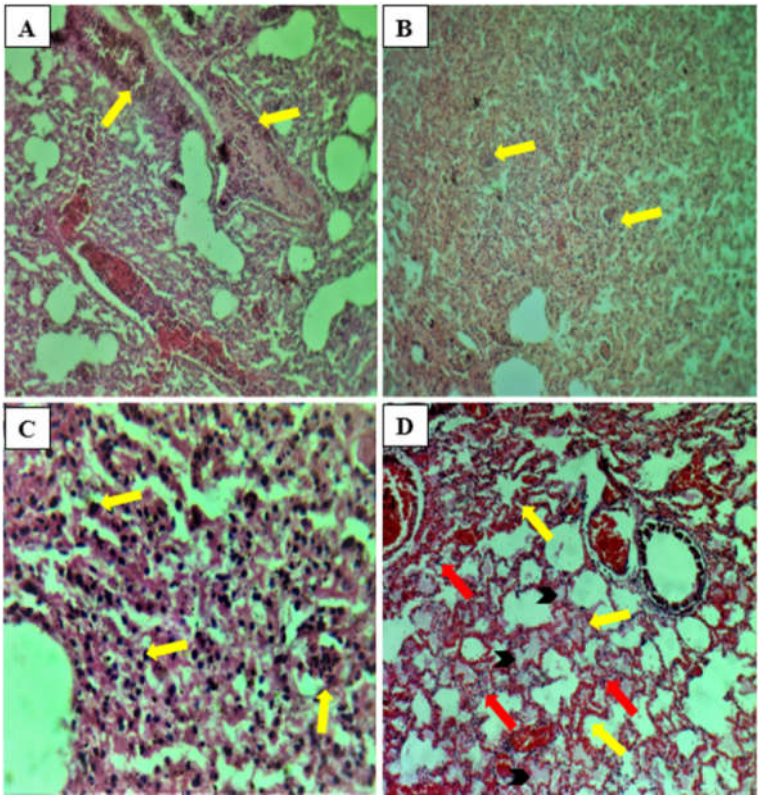

Figure 7: Photomicrographs of lungs. A,B&C/ Severe grade lesion of lungs. Severe grade lesion was characterized by massive hyperplasia of pneumocytes with diffuse infiltration of inflammatory cells (yellow arrows) led to lose normal lung architecture completely. Inflammatory cells aggregated focally or extended in parenchyma. D/ Moderate grade lesion of lungs. Moderate grade lesion was characterized by severe hemorrhage in lung parenchyma with congestion of blood vessels. Thickening of alveolar walls due to alveolar capillaries congestion (yellow arrows), hyperplasia of pneumocytes and infiltration of inflammatory cells (red arrows) was observed. The

thickening of alveolar walls led to narrowing the alveolar spaces, which filled transudate fluid (arrow heads). H&E. A, B &D: 100x, C: 400x

The statistical analysis of lesion scoring results revealed a significant ($p<0.5$) severe lesion of kidney in group G1 compared with groups G3 and G4, and in group G2 compared with groups G3 and G4. Also, a significant ($p<0.5$) severe lesion of the liver was observed in group G4 compared with other *Candida albicans* challenged groups. The significant ($p<0.5$) severe lesion of the lung was observed in groups G3 and G4 compared with group G1 (Table 7).

Table 7: Statistical analysis of lesion scoring of groups G1, G2, G3 and G4 mice.

Group	Lesion scoring mean rank		
	Kidney	**Liver**	**Lung**
G1	21.50^{a}	16.00	10.92
G2	23.25^{a}	11.33	17.25
G3	12.75	16.33	21.75^{c}
G4	11.75	26.42^{b}	20.17^{c}

***** The letter **a** in superscript indicates the significant differences ($p<0.05$) in groups G1 and G2 compared with groups G3 and G4. The letter **b** in superscript indicates the significant differences ($p<0.05$) in group G4 compared with groups G1, G2, and G3. The letter **c** in superscript indicates the significant differences ($p<0.05$) in groups G3 and G4 compared with group G1.
***** n= 8 mice each group

DISCUSSION

A combination of environmental and genetic factors that affect the characteristics of *C. albicans* and effect on the pathogenicity of this bacterium as a result (6, 21, 22, 23). *C. albicans* showed a variation in adhesion and biofilm formation abilities when cultured in different media. Previous researches documented that adhesion and biofilm abilities decreased in *C. albicans* cultured in Sabouraud dextrose agar compared with *C. albicans* cultured RPMI1640 (15).

Candida albicans secreted aspartyl protease as a part of virulence factors in the pathogenicity process this enzyme causes a disruption in host cells to promote invading in or between epithelial cells in the target tissue (24). SAP 8 reflected as a secretory protein to the surrounded medium of the microorganism and play a role mainly in the extensive tissue penetration, while SAP 10 classified as binding structural protein (cell surface protein) (8,11,17,25,26).

This study showed a difference in the expression level of SAP8 genes in different study isolates while the total SAP8 gene expressed more than SAP10 significantly in the RPMI 1640 medium, this agrees with findings of Staniszewska and his colleagues when they test the effects of serum on SAP8 and SAP10 expression (24) and also agrees with (27) who mentioned that the Sap8 overexpressed in the late phase of infection and disagree with other studies which indicate that the level of Sap8 expression in oral infection is decreased while Sap10 gene expression is unchanged in hyphal mutant strain (7, 27).The structural of SAP10 protein with C-terminal sequences (glycol- phosphotidylinositol (GPI)–anchored proteins), make it unable to be secreted from Candida cells and it has an important role as a part of C. albicans cell wall structural protein (7).

Intravenous administration of *C. albicans* can induce multi-organs infection leading to damage of these organs. Kidney is a preferred site in *C. albicans* systemic infection and theses infection may lead to severe damage in kidney parenchyma. According to lesion scoring results, significant severe lesion of kidney was observed in mice injected with *C. albicans* grown in PRMI1640 medium compared with mice injected with *C. albicans* grown in SDA. Also, fungi colonies were observed in mice that injected with *C. albicans* grown in PRMI1640 medium and did not observe in other groups.

However, lesion scoring results showed a significant lesion in liver of mice injected with *C. albicans* grown in laboratory condition and significant severe lesion in lung of mice injected with same fungi, in contrast to results of mice injected with *C. albicans* grown in host condition where the significant severe lesion observed in kidneys. According to histopathology results, *C. albicans* grown in laboratory condition showed a different path of pathogenicity by targeting the liver and lung in comparing with *C. albicans* grown in host condition, where the severe lesion was observed in kidney.

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