

## Kufa journal for Veterinary Medical Sciences

[Kj.vs@uokufa.edu.iq](mailto:Kj.vs@uokufa.edu.iq)

[www.uokufa.edu.iq/journals/index.php/kjvs](http://www.uokufa.edu.iq/journals/index.php/kjvs)

### Antifungal potency of clove (*Syzygium aromaticum*) essential oil extract against induced systemic infection by *Candida albicans* in mice

Ban M. Ali<sup>1</sup>

Ooroba M.S .Ibrahim<sup>2</sup>

Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

\*Corresponding Author: Ooroba Mohammed Saeed Ibrahim E-mail:

[auroba.m@covm.uobaghdad.edu.iq](mailto:auroba.m@covm.uobaghdad.edu.iq) ,

orcid.ID2 : [0000-0002-0682-8621](https://orcid.org/0000-0002-0682-8621)

Co-authors: Ban Manaf Ali E-mail: [Ban.manaf1206h@covm.uobaghdad.edu.iq](mailto:Ban.manaf1206h@covm.uobaghdad.edu.iq)

orcid ID1: [0000-0003-0189-9874](https://orcid.org/0000-0003-0189-9874)

Received date: 29 Jan. 2023 Accepted:(493) 8 Feb. 2023 page: (17-26) Published:30 June. 2023

DOI: <https://doi.org/10.36326/kjvs/2023/v14i111158>

#### Abstract

Fungal infection is a serious health problem, involved with the life threatening mycosis and mortality. Emerging of resistance and limited antifungal drugs against most antifungal agents lead to the requirement for development of effective and alternate strategies to fight fungal infections. The aim of this study is to investigate the antifungal activity of clove oil extract against systemic induced infection *In vivo* by *Candida albicans* in mice. Fifty mice were used to induce systemic infection by injecting 1 ml of  $1 \times 10^6$  *C.albicans* suspension intra-peritoneally for seven days consecutive, then sacrificed to observe the incidence of infection in internal organs (gross lesion) and measure the level of IL-6 and serum creatinine plus histopathological changes. The development of systemic infection with *C. albicans* ( $1 \times 10^6$  cells) was observed daily for 14 days which was accompanied with clinical signs of infection, including weight loss, depression, and ruffled appearance. IL-6 level showed no significant difference in all group before treatment and after inducing infection the level of IL-6 showed significant increase ( $P \leq 0.01$ ) in control positive group while group treated with clove oil 5 mg/kg and 10 mg/kg showed significant decrease in the level compared with infected group and fluconazole treated group . Serum creatinine concentration was within the normal values in all groups ( $p > 0.01$ ) before the infection. There were an increase significantly ( $P \leq 0.01$ ) in the level in all infected mice after 7 days of infection. The serum creatinine values of treated group with clove oil extract 5 mg/kg and 10 mg/kg showed decreased in the creatinine concentration . The present results demonstrated that the essential oil of clove extract rich with eugenol has great antifungal power. This extract can be suggested as antimicrobial and antifungal to treat candidemia and reduce the incidence of mortality rate due to systemic candidiasis.

**Keywords:** *C. albicans*, Clove oil, Eugenol, Gross examination, Interleukin-6, Creatinine, Histopathological changes.

**Introduction** Fungi have been considered to be plants until the end of the 19<sup>th</sup> century, however they are hetero-trophic eukaryotic organisms, nowadays, fungi are gathered in their own kingdom, which is expected to contain more than one- million species, only a small portion of approximately (400) species

have been recognized as human and animals pathogens and the numbers are increasing over time [1].

Systemic fungal infections typically start in the lungs (aspergillosis and other mold from inhalation) or from endogenous flora (candidemia from leakage from the

gastrointestinal tract), and they can spread to many other organs. These infections are medical emergencies and have high mortality rates, especially if proper therapy is impeded. The organisms that cause systemic fungal infection can be divided into two groups: *Aspergillus* and *Candida* species are opportunists, while dimorphic fungi are true pathogens since they can enter and grow in the tissues of a healthy host without showing any signs of a predisposition [2].

Development of resistance in fungal pathogens to the available drugs is an emerging clinical problem in antifungal therapy [3]. Several host, fungal and environmental factors influence the development of resistance. Adaptive phenotypic plasticity, mutations in target genes followed by selection, chromosomal aneuploidy, sexual reproduction and

**Ethical consideration**

The ethics board at Baghdad University approved the study, and each person who took part in the study gave their written approval after being told about the study. This work was done by following the World Medical Association's Code of Ethics for studies that involve individuals.

#### **Preparation of clove oil extract ( *Syzygium aromaticum* )**

The extraction clove oil buds by organic solvent was prepared by using *n*-hexane (95%) which has a high effect in extracting the active components of the herb depending on the technique designated by [6]. That was completed by Soxhlet-apparatus, 30 grams of powder was placed inside the thimble and 95% *n*-hexane 250 ml was put inside the flask. This process has been done for 24 hours by heating temperature reached 40-50 C°. The acquired extract was concentrated on a rotary evaporator at below 40°C. The extracts were weighed to determine the dried yield and then stored at 20°C.

#### **Experimental Animals**

For each test, 50 Albino Swiss female mice (*Mus musculus*) were used. They were put in a plastic cage by themselves. The animals were kept in a room with a temperature between 23 and 25 °C. They had free access to standard pellets and water (ad libitum)..

horizontal gene transfer are the driving forces for emergence of antifungal resistance [4]. Clove is one of the spices that may be used as additives in many foods, particularly in meat processing, to replace chemical preservatives due to their antioxidant and antimicrobial properties, they are commercially used for various medicinal purposes as well as in the fragrance industry. Both clove essential oil and its volatile vapour strongly inhibited the spore germination and mycelial growth of the dermatophytic fungi, including *Candida albicans* and *Trichophyton rubrum* [5].

Therefore, the aim of the study was to evaluate *in vivo* antifungal efficiency of Clove (*S. aromaticum*) buds extracts and using as alternative therapy against systemic infection induced by Pathogenic isolate of *Candida albicans* in mice.

#### **Materials and Methods**

##### **Inducing systemic Infection**

Female mice were infected by *C. albicans*. Once the pilot study has been done, the challenge dose which used to induce infection was  $1 \times 10^6$  CFU/ml of *C. albicans* suspension, and the standardized inoculums prepared according to spectrophotometer. (1 ml )of *C. albicans* was intra-peritoneally administered into the mice for a week, then sacrificed to observe the incidence of infection in internal organs [7].

##### **Experimental designs**

Fifty female mice weighing 20-25 gm, used to perform the experiment of induce systemic infection, were divided equally into five groups (n= 10 each). Administrations of treatment were performed on days 9 after sacrificing and confirming that the infection has occurred.

**Group A:** healthy mice left without infection as negative control.

**Group B:** infected mice left without treatment as positive control.

**Group C:** mice infected and treated orally with 0.1 ml/ 20 gm of clove extract daily for 7 days

**Group D:** mice infected and treated orally with 0.2 ml/ 20 gm of clove extract daily for 7 days

**Group E:** mice infected and treated orally with 0.5 ml/ 20 gm of fluconazole daily for 7 days

#### **Parameters:**

##### **Gross examination**

At the end of the experiment, one animal of each group was sacrificed by using high dose of chloroform. Then, postmortem examination was done for animals to observe the changes that appeared grossly on the tissue before infection, after appearance of lesion and after 7 days of treatment.

##### **Collection of blood**

blood was collected in test tubes with no anticoagulant that allowed to stand and coagulate. Serum was separated from coagulated blood samples by centrifugation at 3000 round per minute (rpm) for 5 minutes and then serum samples were stored in a freezer at -8 °C till use in the creatinine test before infection, after appearance of lesion and after 7 days of treatment [8].

##### **Histopathological Studies**

#### **Results:**

##### **Extraction of clove oil**

Extraction of clove bud with 95% n-hexane revealed a bright yellow color extract with The development of systemic infection with *C. albicans* ( $1 \times 10^6$  cells) was observed daily for 14 days for morbidity and mortality. All mice injected with *C. albicans* resulted in 50 % mortality after 7 days post-infection which is characterized by the presence of abscess and congestion (figure 1). After 7 days of treatment with oil extract of *Syzygium aromaticum* 5

Mice were placed to sleep, and tissue samples were taken. The tissues were washed with PBS, fixed in 10% neutral buffered formalin, and covered with gauze. After the tissues were fixed, they were dehydrated by passing them through 70%, 80%, 90%, and 100% ethyl alcohol twice each for 2 hours, and then they were cleaned with xylene for 1/2 hour. Samples were filled with paraffin wax at 58–60 °C and then covered with more paraffin wax to make paraffin blocks. Using a rotary microtome, sections 5-6um thick were cut, soiled with eosin and hematoxylin stains, and then looked at under a microscopic examination before infection, after infection and after 7 days of treatment [9].

##### **Measurement of tissue IL-6**

Mice were euthanized and tissue biopsies have been taken then tissue was placed in a fridge for freezing for 24 hrs. Tissue homogenate has done to measure IL-6 by ELISA test, specimens were collected before infection, after appearance of lesion and after 7 days of treatment. The test was done according to [10].

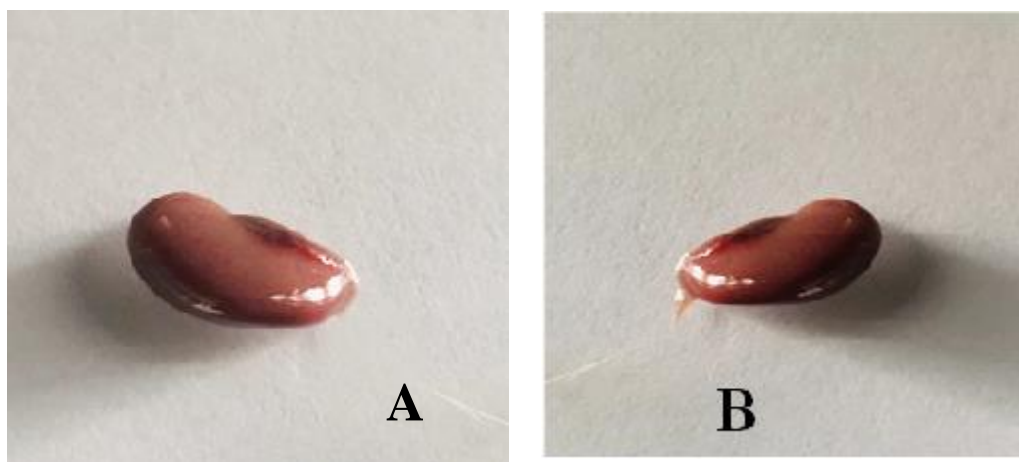
typical clove oil smell and bud, and the percentage of the powder yield 46.6%.

##### **Gross examination**

mg/ml and 10 mg/ml, due to the action of clove oil, namely eugenol, as an antifungal and its potential to produce spores and micelles lysis, the lesions and inflammatory symptoms are gone (figure 2) compared with the group treated with fluconazole 5 mg/ml, which indicated the elimination of lesion after 14 days (figure 3)



**Figure (1):** Gross lesion of kidney of infected mice with *C albicans* shows the presence of abscess with congestion



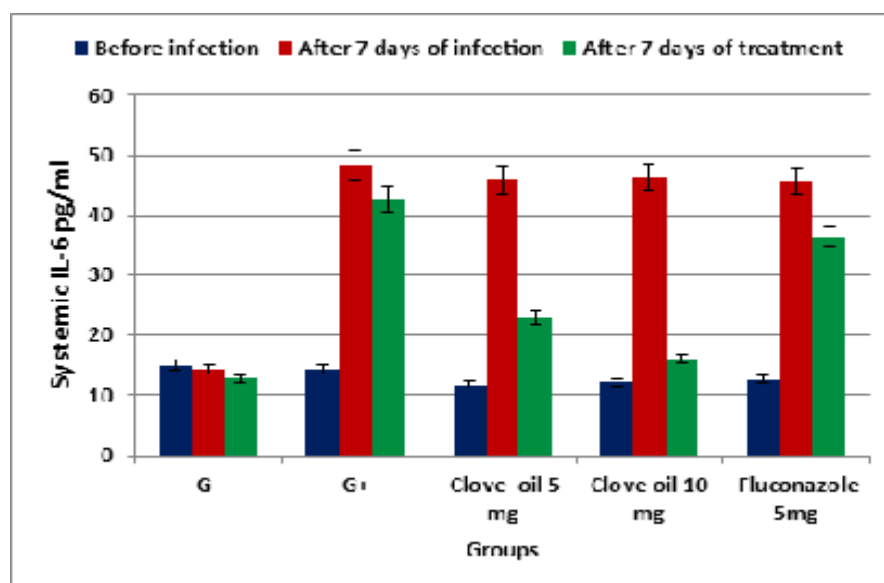
**Figure (2) A:** Kidney infection of mice with *Candida albicans* treated with clove oil 5 mg/kg after 7 days of treatment showed normal appearance with absence of abscess and nodules. **B:** Kidney infection of mice with *Candida albicans* treated with Clove oil 10 mg/kg after 7 days of treatment showed absence of nodules.



**Figure (3):** kidney infection of mice with *Candida albicans* treated with fluconazole 5 mg/kg after 7 days of treatment showed absence of abscess with slight congestion.

#### Measurement of tissue IL-6

The result acquired from the (Figure 4) revealed that there are no significant difference in the level of interleukin-6 in all groups before infection and significant increase after 7 days of inducing infection compared with negative control group. After 7 days, the *Syzygium aromaticum* oil extract-treated group showed a significant reduction ( $P \leq 0.01$ ) in the level of IL-6 compared with fluconazole treated group and positive control group and non-significantly with negative control group. While, the treated group with fluconazole 5 mg/ml after 7 day of treatment still increase with non-significant compared with positive untreated group.

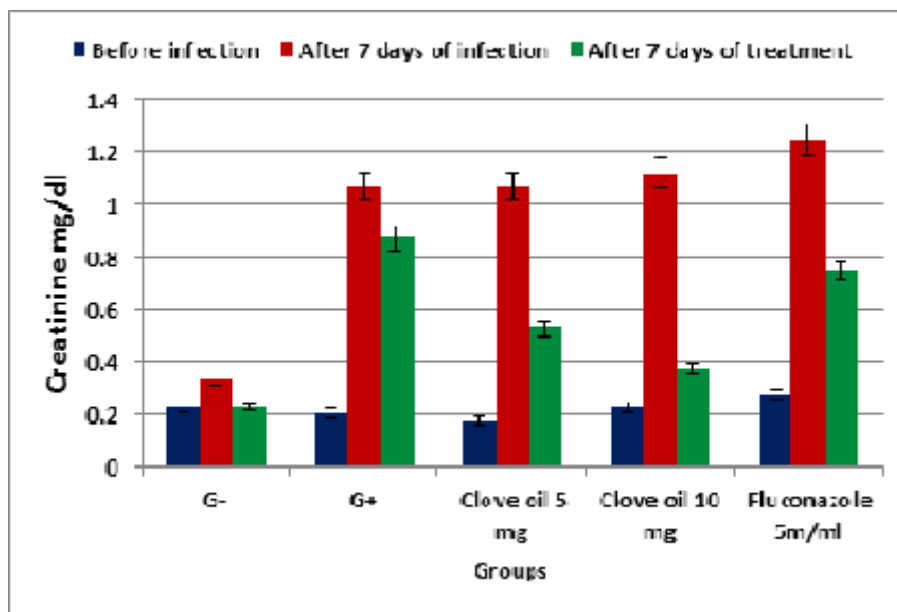


**Figure 4:** Systemic IL-6 (Pg/ml) values of mice in different groups infected with *C.albicans* and treated with Clove oil and fluconazole, or kept without treatment during the course of experiment.

### Blood creatinine measurement

Various levels of mean serum creatinine values in all untreated mice after treated with schedule treatment for 7 days as well as control group are represented in the (Figure 5). It can be seen that serum creatinine concentration was within the normal values in all groups ( $p > 0.01$ ) before the infection. There were an increase significantly ( $P \leq 0.01$ ) in the level of serum creatinine concentration in all infected mice after 7 days of infection except negative control group. The serum creatinine

values of treated group with *Syzygium aromaticum* oil extract 5 mg/ml showed decreased in the creatinine concentration but still high compared with negative control group. While, treated group with *Syzygium aromaticum* oil 10 mg/ml exhibited a significant decrease at ( $P \leq 0.01$ ) in comparison with positive group and non-significantly with negative control group. While, the serum creatinine value of fluconazole treated group showed decrease in the level but still high compared with negative control group.

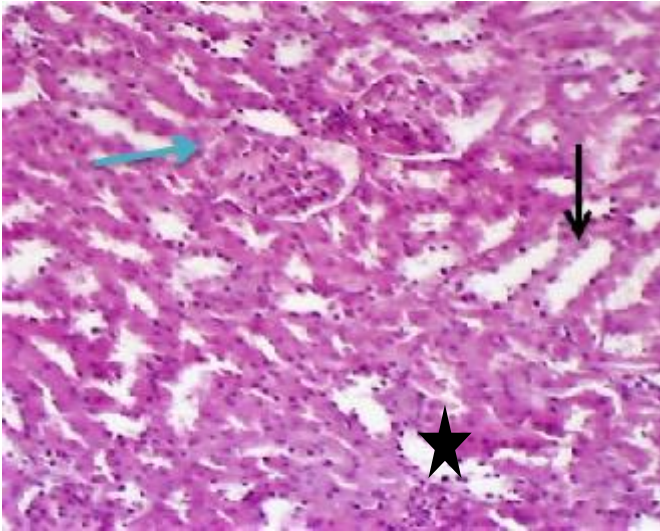


**Figure 5:** Creatinine (mg/dl) values of mice in different groups infected with *C. albicans* and treated with Clove oil and Fluconazole or kept without treatment during the course of experiment.

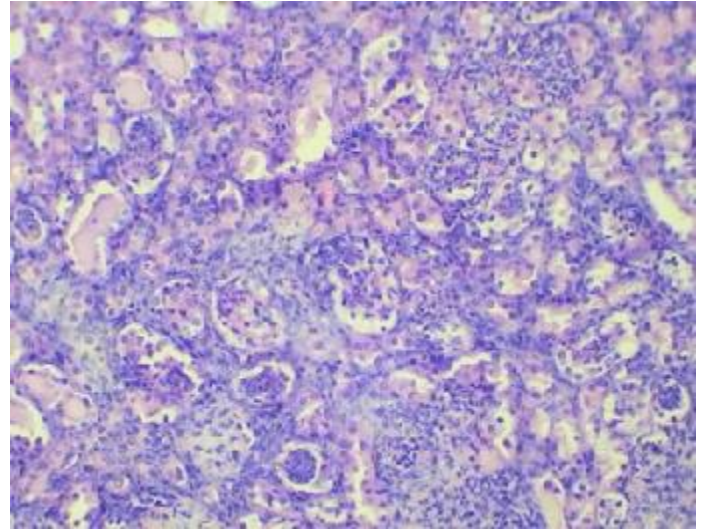
### Microscopically changes

The kidney sections in healthy group showed normal appearance of kidney tissue (figure 6) infected positive group after seven days of infection revealed; glomerulonephritis characterized by intra-tubular infiltration of inflammatory cells, necrosis of lining epithelial cells of renal tubules in cortex area, with thickening of bowman's capsule and atrophy of the glomerular tubules in affected areas also inflammatory cells present. heavy presence of the non-septated hyphae of candida and shows moderate to mild epithelial

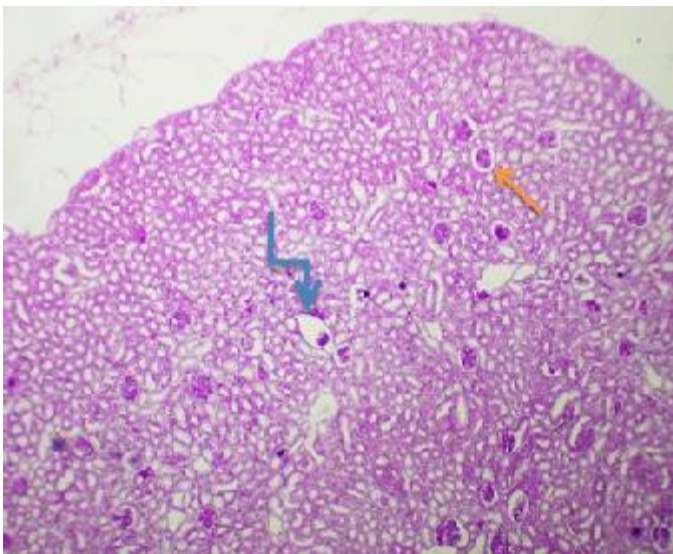
cells degeneration of renal tubules in cortex areas (Figure 7). Group therapy with 10 mg/ml and 5 mg/ml of *Syzygium aromaticum* oil, respectively; the renal tubules in cortex areas appeared predominant normal tubular epithelium, few inflammatory cells of renal tubules in cortex area (Figure 8,9). While the histopathological section of group treated with fluconazole 5 mg/kg showed moderate to mild epithelial cells degeneration of renal tubules in cortex areas. Few lymphocytes infiltrate intertubular tissues (Figure 10).



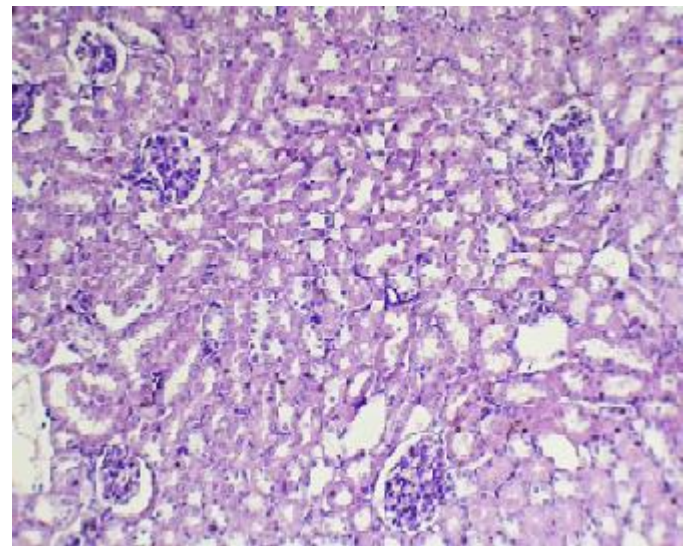
**Figure 6:** Histopathological section of kidney of healthy mice show normal appearance of glomeruli and tubules (H&E stain,10X).



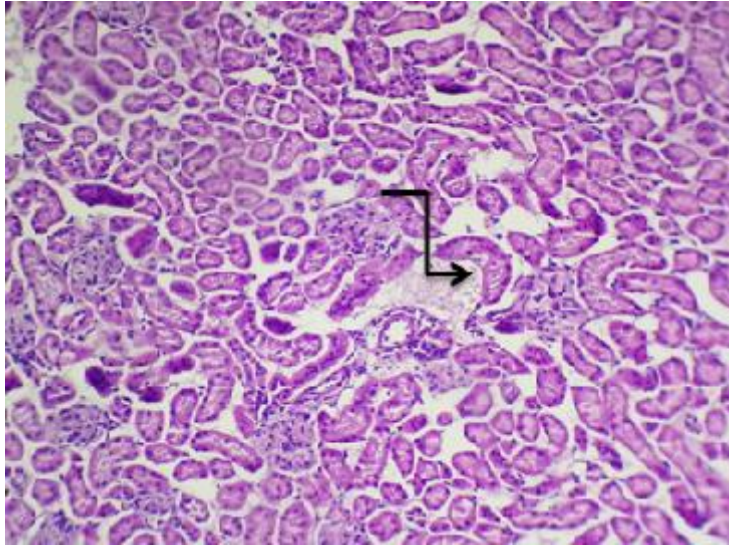
**Figure 7:** Histopathologic section of kidney of infected group shows necrosis of renal tubule in cortex area with infiltration of inflammatory cells and cellular debris (star), with atrophic tubules seen in affected areas (H&E stain, 10X)



**Figure 8:** Histopathologic section of kidney of mice treated orally with clove oil extract 5mg/ml shows normal renal tubules and glomeruli in cortex area (red arrow) with mild vacuolar degeneration of glomerulus surround by normal tubules (blue arrow) (H&E stain, 10X)



**Figure 9:** Histopathological section of kidney of mice treated orally with 10mg/ml shows predominant normal tubular epithelium, few inflammatory cells of renal tubules in cortex area. (H&E stain,10X)



**Figure 10 :** Histopathologic section of kidney mice treated orally with Fluconazole 5 mg/ml shows moderate to mild epithelial cells degeneration of renal tubules in cortex areas. few lymphocytes infiltrate intertubular tissues (H&E stain, 10X).

## Discussion

Kidney is the main organ in which the multiplication of *Candida* happens, yeasts invade the blood vessels walls of both cortex and medulla, causing neutrophil infiltration, renal infection, as distinguished from other organs, is not controlled [11]. Within the first 12 hours, yeast forms considerably multiply and elongate as they extend from the interstitium into tubules and produce germ tubes. Mycelial casts travel into the medulla, settle in the Henle Loop, and trigger an inflammatory response made primarily of mononuclear cells. It has been noted that the kidneys develop granulomatous pyelonephritis and diffusely dispersed abscesses [12]. The ability of the fungus to produce pseudohyphae may be related to the mass-forming property; the mass is composed of granulomatous inflammation, abscess, and necrosis with pyelonephritis [13]. The activity of the essential oils as antimicrobial can be described by the lipophilic properties of their monoterpenoid constituents, these components across through cell wall and cytoplasmic membranes, leading to membrane expansion, improved membrane fluidity and the inhibition of membrane-embedded enzymes [14]. Due to their similar molecular structures, which contained around 80% of the components of clove oil, eugenol clove oil have strong

biological effects as a result of the presence of an alkyl group connected to Carbon 4 and an OH at Carbon 1, which play a crucial role in creating covalent connections with microorganisms and inhibiting their growth, Eugenol action could be referred to as being structural in nature [15]. After 7 days of infection, *C. albicans* disseminated to affect vital organs mainly kidney thus, inducing inflammatory process which is characterized by an inflammatory cytokine such as interleukin-6 which is secreted in response to microbial infections in body, the potential contribution of IL-6 in the proliferation of glomerulonephritis assumes that this cytokine may trigger kidney mesangial cells proliferation. Certainly, IL-6 transgenic mice reveal mesangial proliferative glomerulonephritis [16].

Numerous studies have examined the anti-inflammatory properties of clove and eugenol, finding that these ingredients can modulate a number of inflammatory markers, including COX-2, nitric oxide iNOS and prostaglandin E2, leukotriene C4, and mast cell degeneration [17, 18]. Clove essential oil (EO) blocks the production of these cytokines [19]. One typical mechanism for activating various damaging factors or the threat of further harm is the beginning of the pro-

inflammatory cell-stress program [20]. The kidney sections in infected positive group after seven days of infection revealed; glomerulonephritis characterized by intra-tubular infiltration of inflammatory cells, necrosis of lining epithelial cells of renal tubules in cortex area, with thickening of bowman's capsule and atrophy of the glomerular tubules in affected areas also inflammatory cells present. The hydrophobicity of the EOs, which causes them to partition into the lipid bilayer of the cell membrane, may be partially to blame for the activity. As typical lipophiles, EOs pass through the cell wall and cytoplasmic membrane, disrupt the structure of the various layers of polysaccharides, fatty acids, and phospholipids, and permeabilize them, this causes an alteration in permeability and a subsequent leakage of cell contents. As a result of tissue breakdown, the abundant enzyme creatinine flows from the heart, the musculoskeletal system, the brain, and the kidney. At 48 and 72 hours after infection, mice infected with the highly pathogenic strain

## References

- [1] De Hoog GG, Gene J, Figueras MJ. Atlas of Clinical Fungi. A.S.M. Press; 2000.
- [2] Colombo AL, de Almeida Júnior JN, Slavin MA, Chen SC-A, Sorrell TC. Candida and invasive mould diseases in nonneutropenic critically ill patients and patients with haematological cancer. *Lancet Infect Dis*. 2017;17(11):e344–e356.
- [3] Berman J, Krysan DJ. Drug resistance and tolerance in fungi. *Nat Rev Microbiol*. 2020;18(6):319–331.
- [4] Hokken MWJ, Zoll J, Coolen JPM, Zwaan BJ, Verweij PE, Melchers WJG. Phenotypic plasticity and the evolution of azole resistance in *Aspergillus fumigatus*: An expression profile of clinical isolates upon exposure to itraconazole. *BMC Genomics*. 2019;20:13.
- [5] Chee HY, Lee MH. Antifungal activity of clove essential oil and its volatile vapour showed a considerable rise in blood creatinine [21].
- Due to eugenol's lipophilic nature and accumulation in the phospholipid bilayer, the antifungal activity of eugenol has been linked to disruption of the fungal membrane structure. This interaction influences the fluidity and permeability of fungal membranes as well as the activity of crucial proteins or enzymes that are membrane-bound [22]. The increases in serum creatinine levels indicate acute or chronic renal dysfunction [23].
- ## Conclusions
- In this work, the activity of *Syzygium aromaticum* oil extract as antifungal against systemic *Candida albicans* has been revealed. The essential oil of clove extract rich with eugenol demonstrated great antifungal power. This extract can be suggested as antimicrobial and antifungal to treat candidemia and reduce the incidence of mortality rate due to systemic candidiasis.
- ## Conflict of interest
- The authors have no conflict of interest against dermatophytic fungi. *Mycobiology*. 2007;35(4):241–243.
- [6] Ishaq AQ, Syed MI, Khan MA, Zia MA. Characterising and optimising antioxidant and antimicrobial properties of clove extracts against food-borne pathogenic bacteria. *Food Sci Biotechnol*. 2019;28(4):1165–1172.
- [7] Latifah-Munirah B, Himratul-Aznita WH, Mohd Zain N. Eugenol, an essential oil of clove, causes disruption to the cell wall of *Candida albicans* (ATCC 14053). *Front Life Sci*. 2015;8:231–240.
- [8] Hinkle J, Cheever K. Brunner & Suddarth's Handbook of Laboratory and Diagnostic Tests. 2nd ed. Philadelphia: Wolters Kluwer Health, Lippincott Williams & Wilkins; 2014. Creatinine, serum; p. 198.
- [9] Suvarna SK, ed. Atlas of Adult Autopsy: A Guide to Modern Practice. London: Springer; 2016.

- [10] Bodzon-Kulakowska A, Bierzynska-Krzysik A, Dylag T, Drabik A, Suder P, Noga M, Jarzebinska J, Silberring J. Methods for samples preparation in proteomic research. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2006;849(1-2):1-31.
- [16] Horii Y, Muraguchi A, Iwano M, et al. Involvement of IL-6 in mesangial proliferative glomerulonephritis. *J Immunol.* 1989;143(12):3949–3955.
- [11] MacCallum DM, Odds FC. Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses.* 2005;48(3):151–161.
- [12] Korkeas F, Favoretto RL, Bróglia M, Silva CA, Castro MG, Perez MDC. Xanthogranulomatous pyelonephritis: Clinical experience with 41 cases. *Urology.* 2008;71(2):178–180.
- [13] Lin L, Ibrahim AS, Baquir B, Palosaari A, Spellberg B. Luminescent-activated transfected killer cells to monitor leukocyte trafficking during systemic bacterial and fungal infection. *J Infect Dis.* 2011;205(2):337–347.
- [14] Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils—a review. *Food Chem Toxicol.* 2008;46:446–475.
- [15] Ghasemi G, Fattahi M, Alirezalu A, Ghosta Y. Antioxidant and antifungal activities of a new chemovar of cumin (*Cuminum cyminum* L.). *Food Sci Biotechnol.* 2019;28(3):669–677.
- [17] Lee YY, Yang SF, Ho WH, Lee YH, Hung SL. Eugenol modulates cyclooxygenase-2 expression through the activation of nuclear factor kappa B in human osteoblasts. *J Endod.* 2007;33(10):1177–1182.
- [18] Magalhães CB, Riva DR, DePaula LJ. In vivo anti-inflammatory action of eugenol on lipopolysaccharide-induced lung injury. *J Appl Physiol.* 2010;108(4):845–851.
- [19] Rodrigues TG, Fernandes Jr A, Sousa JPB, Bastos JK, Sforcin JM. In vitro and in vivo effects of clove on pro-inflammatory cytokines production by macrophages. *Nat Prod Res.* 2009;23(4):319-326.
- [20] Gusev EYU, Chereshev VA. [Systemic inflammation: Theoretical and methodological approaches to description of general pathological process model. Part III. Prerequisites of non-syndromic approach]. *Patologiya, fiziologiya, eksperimental'naya, terapiya.* 2013;3:3–14. (in Russian)
- [21] Lewis SL, Bucher L, Heitkemper MM, Harding MM, Kwong J, Roberts D. *Medical-Surgical Nursing: Assessment and Management of Clinical Problems.* 10th ed. St. Louis: Elsevier Health Sciences; 2016. p. 1025.
- [22] Wang C, Zhang J, Chen H, Fan Y, Shi Z. Antifungal activity of eugenol against *Botrytis cinerea*. *Trop Plant Pathol.* 2010;35(2):137–143.
- [23] Pandya D, Nagrajappa AK, Ravi KS. Assessment and correlation of urea and creatinine levels in saliva and serum of patients with chronic kidney disease, diabetes, and hypertension: A research study. *J Clin Diagn Res.* 2016;10(9):ZC01–ZC04.