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Protective role of Garlic Oil Pre-and Post-Infection of Mycoplasma agalactia in Albino mice

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Abstract
The present study aimed to evaluate the effect of garlic oilextraction (GOE) on interleukin18 (IL18) in albino mice before and after infection with mycoplasma agalactia. Thirty Balb/c mice were allocated into three groups:1) G: mice treated with 100 mg \kg of garlic oil orally for30 before and after infection with mycoplasma agalactia: 2) P: mice infected with mycoplasma agalactia for 30 days without any treatment; 3) N: control group.The isolation was obtained by culturing milk samples from goats and sheep.the mice infected with mycoplasma agalactiaintraperitoneally.The clinical signs are reported from infected mice daily and after a month of infection the mice are euthanized and IL18 was measured using, indirect enzyme-linked immunosorbent assay (ELISA), in addition to the male reproductive system histopathological examination. The results showed the effectiveness of garlic oil in reducing infection, as there was a significant increase in the level of IL18 in mice treated with garlic oil compared to the control group. The IL18 was 273.45 in the treated group with garlic oil, compared to 40.86 in the appositive control group and 7.49 in the negative control group.Histopathological examination of the testis showed subcapsular infiltration of inflammatory cells and degeneration.

Keywords: mycoplasma agalactia, garlic oil, IL18, Elisa

INTRODUCTION

One significant illness that affects small ruminants is called contagious agalactia (CA), which results in a significant decrease in milk output or even its termination. CA is considered a notifiable illness by the World Organization for Animal Health (WOAH, formerly known as OIE)(Chazelet al.,2010), It is found throughout the world, especially in nations with advanced dairy sheep husbandry; it is also endemic to Southern Europe, the Middle East, Asia, and North Africa.(Bohachet al.,2021).According to Jaÿ and Tardy (2019), the global rise in small ruminant milk production has made it even more significant in recent times.Nonetheless, Mycoplasma putrefaciens, Mycoplasma capricolum subsp. capricolum, and Mycoplasma mycoides subsp. capri have been found to cause a sporadic clinically similar syndrome, especially in goats (Bohachet al., 2021; Corrales et al., 2007). Mycoplasma agalactiae (Ma) is reported to be the main causative pathogen for CA cases. A subclinical version of the disease caused by the pathogen M. agalactiae can persist in animals for several years. According to Jafarizadeh et al. (2017), animals that are affected by agalactia during the lambing season exhibit clinical signs, which impact over 70% of livestock.According to Kumar (2014), the duration of incubation varies based on the mode of transmission, immunological status of the host, and severity of the infecting strain. It can last anywhere from a few days to several weeks or even upwards of two months.As a result, chemotherapy and biosafety measures (i.e., culling or isolating diseased animals, maintaining cleanliness, etc.) are the primary means of managing CA as there are currently no commercially available vaccinations with proven efficacy (Agnoneet al., 2013).Right now, natural products—of which plant-derived natural products are the representativeare gaining a lot of attention as potent therapeutic agents for treating human disorders (Alinezhadet al., 2011).

The phrase "plant-derived natural products" mostly refers to the secondary metabolites of plants, which are abundant in nature, have a wide variety of forms, and include a wide range of potent antibacterial constituents, including organic acids, polyphenols, flavonoids, alkaloids, and essential oils. since they work well and provide a range of antibacterial defences against the growth of drug resistance. As complex mixes of several volatile ingredients such as alcohols, esters, monoterpenes, sesquiterpenes, and ketones, essential oils are aromatic, oily liquids with an oily texture. Studies demonstrate the significance of essential oils in plants' defence against bacteria, fungus, and pests (HarkatMadouriet al., 2015).The subterranean bulb of the Allium family, Liliaceae, is known as garlic (Allium sativum L.). In the ninth century, it was brought to Japan and South Asia from its original homelands in Central Asia and the Mediterranean. Furthermore, it is currently extensively grown

throughout the world. One well-known plant that can be used for both food and medication is garlic. From ancient times, it has been widely used to treat and prevent headache, tumors, diarrhea, and other illnesses(Nagini, 2008).The unique flavor and pharmacological benefits of garlic are attributed to its over 33 distinct organic sulfur compounds(DeGreefet al., 2020).The essential oil of garlic currently contains a wide variety of medicinal components. Garlic essential oil is incredibly rich in phenolic compounds, steroidal saponins, essential amino acids, saponin ligands, and other non-sulfur chemicals in addition to organic sulfides like DADS and DATS(Amagase, 2006).In 1995, interleukin (IL) 18 was first shown to be a proinflammatory cytokine that activates type 1 helper T cells to generate interferon (IFN)-γ(Okamura et al.,1995).Many immune cells, including macrophages, dendritic cells, and monocytes, produce IL-18, which is known to be a crucial modulator of both the innate and acquired immune systems(Nakanishi et al .,2001).IL-18 is a member of the IL-1 superfamily that is mostly produced by macrophages but can also be produced by other cell types. It stimulates different cell types and has a variety of pleiotropic effects. Type 1 reactions are aided by the proinflammatory cytokine IL-18. When microbial components like lipopolysaccharide (LPS) are infected, it triggers cell-mediated immunity in conjunction with IL-12. When IL-18 and IL12 are combined, they operate on CD4, CD8 T cells, and NK cells to stimulate the production of IFNγ, a type II interferon that is crucial for activating macrophages and other cells. Combining IL-18 and IL-12 has been demonstrated to increase B cell synthesis of IgG2a while inhibiting IL-4-dependent IgE and IgG1 production(Garlandaet aL.,2009). Importantly, without IL-12 or IL-15, IL-18 does not induce IFNγ production but plays an important role in the differentiation of naive T cells into Th2 cells and stimulates mast cells and basophils to produce IL-4, IL-13, and chemical mediators such as histamine .(Yasuda et al.,2019).

2. MATERIALS AND METHODS

2.1. Ethics Statement

Tests were directed as prescribed by the guidelines of the college of veterinary medicine. University of Baghdad reviewed and approved all procedures involved in the current study under the agreement number 369\P.G. dated on February 18,2024.

2.2. Single Garlic Essential Oil Extraction:

gelatinous capsules of garlic oil (NOW®'s Garlic Oil is extracted and concentrated from the bulb of Allium sativum.

2.3. Animals, mycoplasma isolate and inoculum preparation

Thirteen adult Albino mice were selected fromAlrazi centre microbiologically and serologically negative for Ma, other mycoplasmas, bacteria, or fungi (Assunção et al., 2004). The field strain 7MAG of Ma was isolated in 200 from a milk sample of a goat from an infected herd in different regions of Bagdad (Abo Ghraieb,Altaji) with clinical signs of contagious agalactia including mastitis and arthritis.Ma microorganisms were grown in milk samples initially, then into PPLO broth and on PPLO agar media in accordance with normal protocols to prepare the inoculum. An incubator with 5% CO2 and humidity was used to incubate the infected media at 37 º C. Every day, the broths were checked for growth indicators, such as fluctuations in pH that could be detected by color changes or changes in the turbidity of the medium. A typical "fried egg" look of mycoplasma colonies was observed on the plates after two to three days under 100X magnification**.** (Kizil and Ozdemir, 2006).

2.4. Experimental design

A 0.1 ml-inoculum containing 1×10^4 colony-forming units (cfu) of Agalactiawas injected intraperitoneally. Infected mice were randomly assigned to two groups of ten animals each, the positive control which infectedwithout treatment and the other group which immunization with garlic oil100 mg\kg orally for 30 days and then infected with agalactiaand euthanized at 30 days post-infection. The remaining ten mice served as uninfected controls. Animals were placed in cages, fed a commercial diet, and subjected to regular clinical and serological examinations.

2.5. Statistical Analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of different factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significantly compare between meansin this study.

2.6. ELISA

The serum samples were obtained from the 30 blood samples from mice brought to the laboratory by centrifuging at 3000 rpm for 5 minutes.

The serum samples were tested using ELISA kit (www.elkbiotech.com) was performed for quantifying IL18 in infected and control groups according to the manufacturer's instructions. The optical density (OD) values were read at 450 nm using a plate reader (Biotechnology CO., Ltd., Wuhan, China).

2.7.Histopathological Examination

Animals were scarified by an overabundance of anesthesia following a 30-day MG challenge (Chlorophorm, HiMedia, India). Samples of testes were preserved in 10% formalin for a duration of 72 hours. Treatment of graded ethyl alcohols (70–80%, 90%–100% twice) and the subsequent removal of xylene twice are part of the histological preparation. Next, block using a microtome at 5 meters and liquid paraffin at 56 degrees Celsius. Next, eosin and hemophthalein are used for staining.(Batah and Mirhish , 2019)

3.RESULTS

3.1. Bacterial culture and isolation

Mycoplasma colonies were grown using a pleuropneumonia like organism (PPLO) medium. The growing organism colonies had a look like to a fried egg, with a matte center and a white to colorless aura halo (Fig. 1).

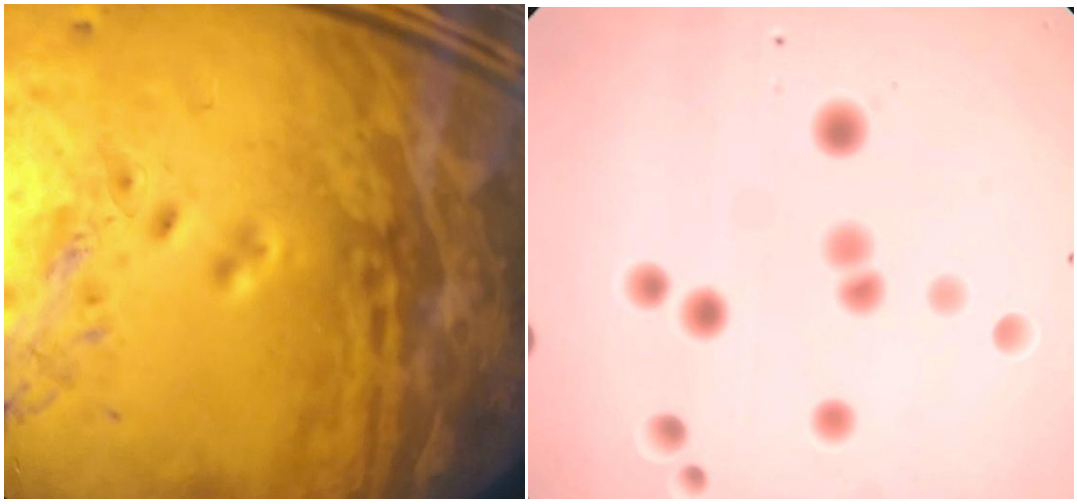

Fig.1. Fried egg appearance of Mycoplasma colonies growing on medium (PPLO Medium) using a light microscope

3.2.Static

Table 1: Comparison between difference groups in IL-18 Conc.

Group	Mean ±SE of IL-18 (ng/ml)
Negative control	7.49 ±2.25 C
Positive control	40.86 ±4.46 BC
Garlic/ Before	47.71 ±6.66 B
Garlic/ After	273.45 ±37.65 A
L.S.D.	39.376 **
P-value	0.0001
Means with different letters in the same column are significantly different. , ** (P≤0.01).	

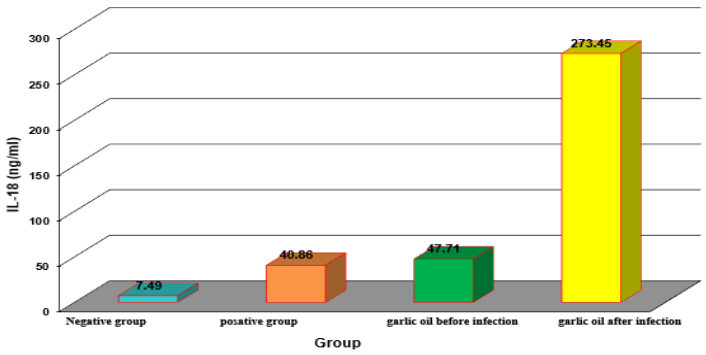

Figure 2: Comparison between difference groups in IL-18 Conc.

3.3.The histopathological examination of tissue sections from different organs treated with Garlic pre and post-challenge with Mycoplasma agalactia compared with positive control;

3.3.1.In positive group

were mice infected with Mycoplasma agalactia; the microscopic changes of epididymis revealed severe degeneration of ductal lining-epithelium (Figure-1&2), sometimes necrotic cells represented by hyperchromatic nuclei and more eosinophilia, most tubules appeared empty from sperms the present sperms were mostly necrotized with presence of cellular and inflammatory debris, there was infiltration of inflammatory cells mainly from mononuclear cells in interstitial between tubules and congestion of blood vessels (Figure-3). Atrophy of seminiferous tubules (Figure-4) was prominent in tissue sections of testis; characterized by decreased number of spermatogonia (primary and secondary) also necrosis and increased eosinophilia of spermatids (Figure-5).

3.3.2.After treatment with garlic oil and challenged with Mycoplasma agalactgia

there was normal spermatogenesis as shown in testicular tissues of mice treated with garlic oil100 mg\kg orally for (time) (Figure-6&7) represented the normal morphology steps of spermatogonial cells, meiosis as hyperchromatic germinal layer, hypertrophy of primary and secondary cells with enlarged spermatids. The epididymis appeared with normal lining epithelium and their lumen contain sperms (Figure-8&9).

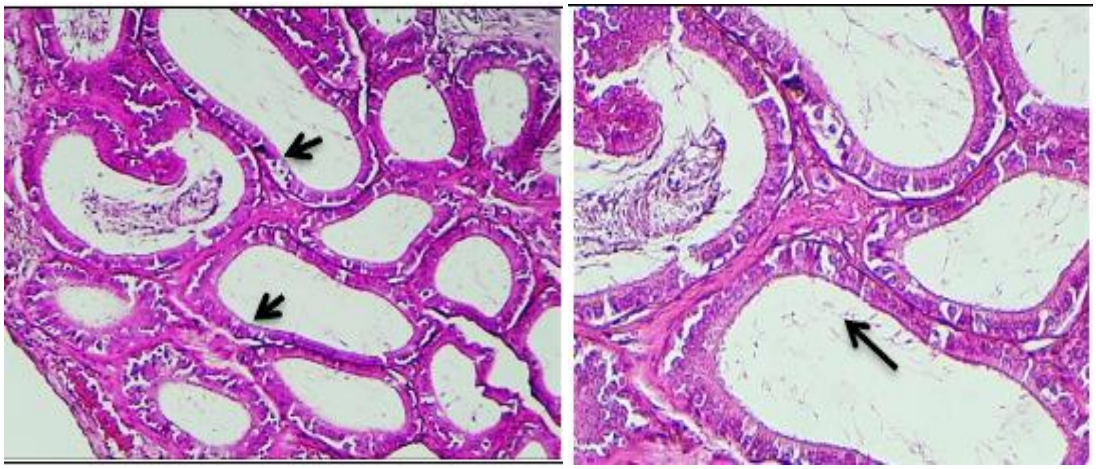

Figure 1: Histopathological section inEpididymusof positive group; shows vacuolar degenerated epithelium (arrow) of ductul tubules. (H&E stain, 200X).

Figure 2: Histopathological section in Epididymus of positive group; high magnification of figure-1 shows vacuolar degenerated epithelium of ductul tubules and few sperms in lumen (arrow). (H&E stain, 400X).

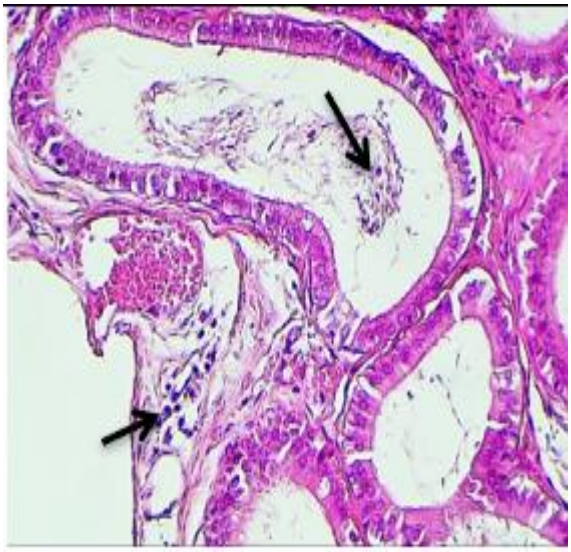

Figure 3: Histopathological section in Epididymus of the positive group; shows subcapsular infiltration of inflammatory cells (arrow) and congestion of blood vessel, the ductul lumen contain w sperms appeared necrotic and degenerated (arrow). (H&E stain, 400X).

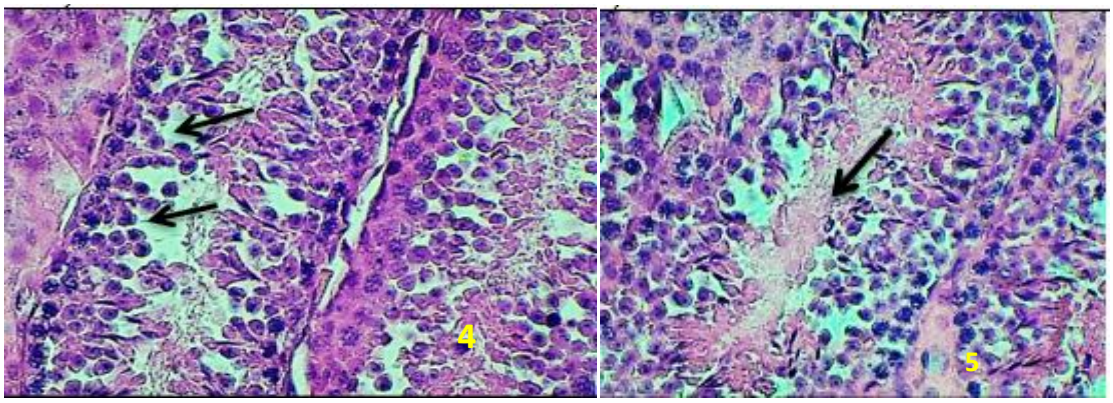

Figure 4: Histopathological section in the testis of positive group; shows atrophy of spermatogonia with a small size of their nuclei (arrow). (H&E stain, 400X).

Figure 5: Histopathological section in testis of positive group; shows atrophy and eosinophilia of degenerated spermatogonia and necrotic spermatids (arrow). (H&E stain, 400X).

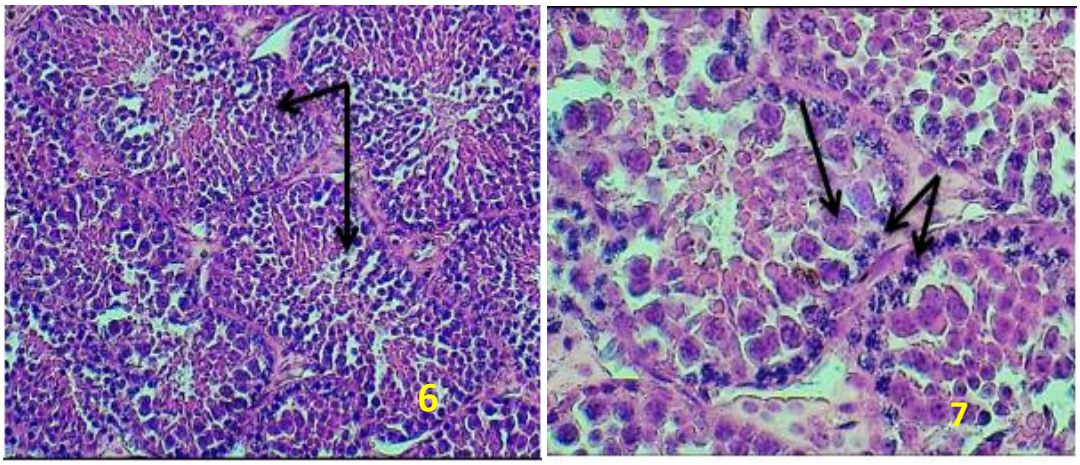

Figure 6: Histopathological section in testis of Garlic group (garlic treatment); shows multi seminiferous tubules (arrow) with normal spermatogenesis. (H&E stain, 200X).

Figure 7: Histopathological section in testis of Garlic group (garlic treatment); shows meiosis of germinal cells (arrow) in basal layer and hypertrophy spermatogonia (primary and secondary). (H&E stain, 400X).

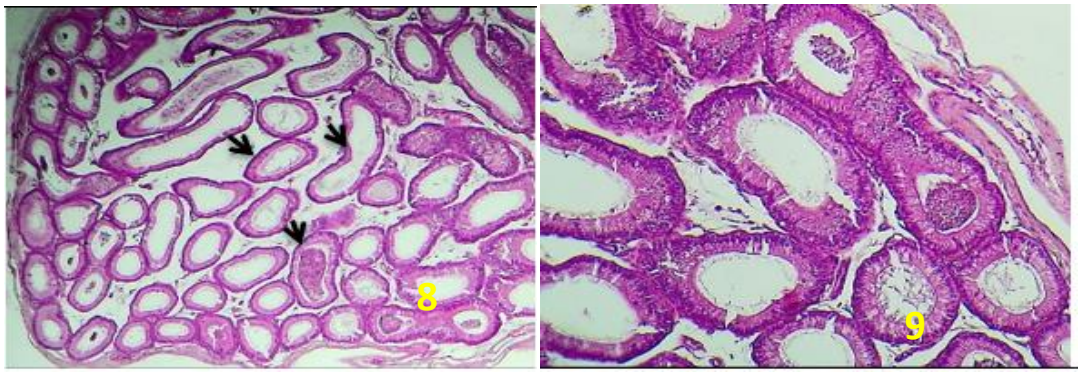

Figure 8: Histopathological section in Epididymus of Garlic group (garlic treatment); shows normal high cuboidal epithelium of ductul tubules (arrow). (H&E stain, 100X).

Figure 9: Histopathological section in Epididymus of Garlic group (garlic treatment); high magnified from figure-3 shows normal lining epithelium of tubules. (H&E stain, 200X).

4. DISCUSSION

Goat farms suffer heavy financial losses due to contagious agalactia, which kills young animals, causes miscarriages in pregnant goats, and, most significantly, reduces milk supply(ÖztürkandYaman, 2024).To diagnose M. agalactiae, a variety of samples are used, including milk, blood, serum, nasal swabs, joint fluid, and vaginal swabs(Lin et al., 2022). Mycoplasma agalactia isolates from goats and sheep milk samples with and

without mastitis.This a study conducted in and around Baghdad approved with Kızıl and Ozdemir (2006) in Elazığ, they collected milk samples from 47 goats showing symptoms of fever, mastitis, and arthritis, as well as from 20 asymptomatic goats. They investigated the samples using both bacteriological and molecular methods and reported that 17 were diagnosed with M. agalactiae using both methods. The samples were subjected to bacteriologic culture and molecular investigations. isolated Mycoplasma spp. colonies on PPLO agar from goats showing mastitis and arthritis symptoms, as well as from the internal organs of deceased animals. This indicates that Mycoplasma spp. can be easily isolated from samples taken from goats exhibiting clinical symptoms of contagious agalactia. Subsequent isolations and studies, such as those by Al-Graibawiet al. (1989) and Hasso (1994), further confirmed the pathogen's prevalence and highlighted the challenges posed by its diagnosis, which requires sophisticated laboratory techniques due to the organism's fastidious nature (Khezriet al.,2015). In this study, the serological diagnosis of the infection was performed using ELISA. To evaluate a possible influence of IL18serum levels in the immunopathogenesis ofM.agalactia in albino mice and controls was performed in this study. It was found that the level of IL18 in the negative group 7.49, while after infection with M.agalactiaincreased to 40.86 in the positive group because the IL-18 is evolving as a major pro-inflammatory cytokine with implications for a role in inflammatory and infectious diseases,this increase because IL18 is released following inflammasome activation (Hu,2017).Stressful events produce IL-18 (i.e., bacterial or neurogenic signals). According to some theories, this situation can result in a cycle of IFN-γ/IL-18 production that is reinforced by the release of IL-18 brought on by stress(Yumotoet al., 2002).Once IL-18 has caused a wave of IFN-γ synthesis, freshly produced IFN-γ can now cause monocytes and macrophages to produce more interleukin-1 converting enzyme (ICE) activity(Allen et al., 2022). In the presence of continued IL-18 production, increased ICE activity probably results in more processed IL-18, leading to more lymphocyte IFN-γ production and more macrophage ICE activity. Thus, IL-18 promotes not only IFN-γ synthesis but also participates in its overall activities(Yamanishiet al., 2019). Based on statistical analysis, the administration of single-clove garlic essential oil extract in the test showed a significantly different result. The treatment groups with single-clove garlic essential oil extract weresignificantly larger than thenegative control group.Garlic has immunomodulatory properties that are mediated by its capacity to control cytokine production and trigger immunological response by promoting immune cells and antibody release(Arreola et al., 2015).Garlic has anti-inflammatory and anti-arthritic properties because of its capacity to inhibit NF-κBsignaling(Ban et al., 2009).Garlic contains several different aliphatic sulfides, ajoene, and allicin as its primary antimicrobial organosulfur components. As opposed to yielding a pure compound, the extraction process concentrates a specific component. Allicin-rich product can be obtained by extracting garlic using ethanol or water and then condensing the extract. Noticeably, the yield of ethanol is higher than that of water. (Fujisawa et al., 2008).Fever, mastitis, conjunctivitis, arthritis, and pneumonia are among the first clinical symptoms that point toward a diagnosis(Jay and Tardy, 2019).There are sporadic reports of genitalia, like vaginitis, salpingitis, metritis, testicular degeneration, and neurological disorders.The histopathological section of the testes of male albino mice after 30 days of infection with M.agalactiashowed severe degeneration of ductal lining-epithelium in Epididymisof positive group and this approved by (Rosales et al.,2017). there was normal spermatogenesis as shown in the testicular tissues of mice treated with garlic oil, garlic essential oil exerts anti-inflammatory effects through dysregulation of IL-10 and reduction of IL-12, preventing IL-12 from binding to its receptors on T and NK cells and, thus, inhibiting IFN-γ production (Hodge et al., 2002). Researchers discovered that certain sulfur-containing compounds in garlic essential oil might raise the amount of glutathione in cells and improve the functions of catalase (CAT), glutathione peroxidase, and superoxide dismutase (SOD) in cells. ROS elimination is facilitated by a number of vital defense systems in live cells. They work as antioxidants as a result.(Chen et al., 2018).he strong anti-inflammatory properties of garlic essential oil are also attributed to its antioxidant action, as numerous prior research have demonstrated. The principal mode of action involves impeding the synthesis of pro-inflammatory cytokines, NFkB activation, inflammatory prostaglandins, thromboxanes, and inducible NO synthase(Seneret al., 2007).Furthermore, some sulfides found in garlic essential oil prevented lipopolysaccharide-activated macrophages from producing NO, prostaglandin E2, or the pro-inflammatory cytokines tumor necrosis factor, interleukin-1b, and interleukin-6(Lee et al., 2012). Studies have also looked at the anti-inflammatory properties of garlic essential oil and the possible molecular mechanism of DADS (diallyldisulfide) on acute pancreatitis and associated lung damage in mice(Mathan Kumar and Tamizhselvi, 2020).

CONCLUSIONS

Research has demonstrated that the use of garlic oil can lower bacterial loads, improve clinical symptoms, and alter immunological responses. These results point to the possibility of using garlic oil as a helpful adjuvant treatment for bacterial infections, providing a viable substitute for traditional antibiotics and assisting in the fight against antibiotic resistance.

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Novelty statement
This study focuses on the immunological role of garlic oil in protecting againstmycoplasma agalactiainfection, as IL18 was measured in groups of albino mice infected with M.agalactiaafter isolation from sheep and goat milk, In addition to that, The effect of mycoplasma on the male reproductive system was studied histopathologically.

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