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RESEARCH ARTICLE

Silymarin Antibacterial Efficacy against some Isolated Bacterial Strains from Pneumonic Sheep - Vitro Study

Hany Hassan[1*], Ahmed Kamr[1], Walid Mousa[2], Ramiro Toribio[3], Abdel Nasser El-Gendy[4], Hadeer Khaled[1], Abdelsalam Elkholey[1] and Ali Arbaga[1]

[1]Department of Medicine and Infectious Diseases (Animal Internal Medicine), Faculty of Veterinary Medicine, University of Sadat City, Sadat City 32897, Egypt
[2]Department of Medicine and Infectious Diseases (Animal Infectious Diseases), Faculty of Veterinary Medicine, University of Sadat City, Sadat City 32897, Egypt
[3]College of Veterinary Medicine, the Ohio State University, Columbus, OH 43210, USA
[4]Medicinal and Aromatic Plants Research Department, Pharmaceutical and Drugs Research Institute, National Research Centre, Cairo 12622, Egypt
*Corresponding author: hanyhassan1959@gmail.com

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ABSTRACT

Pneumonia is a common disease in sheep flocks that causes severe economic losses and high mortality rate among sheep due to high resistance to ordinary standard antimicrobial treatment protocols. This study was to assess silymarin efficacy as an antibacterial agent against some isolated bacterial strains from twenty-five pneumonic sheep *in vitro*. Nasopharyngeal swabs were collected from sick sheep, and placed in a nutrient and pleuro pneumonia like organism broth (PPLO) for bacterial isolation. *P. aeruginosa*, *S. aureus*, *E. coli*, and *M. ovipeumoniae* were isolated bacterial species confirmed by PCR using specific genes. The antibacterial activity of silymarin against *P. aeruginosa*, *S. aureus*, and *E. coli* was evaluated using the well-diffusion technique. It was shown that the minimum therapeutic dosage of silymarin was not less than 280 mg/ml against *P. aeruginosa*, whereas chloramphenicol had little effect. Chloramphenicol exhibited more antibacterial activity against *S. aureus* and, *E. coli* compared to different concentrations of silymarin. The microbroth dilution method determined the minimum inhibitory concentration (MIC) of silymarin against *P. aeruginosa*, *S. aureus*, *E. coli*, and *M. ovipeumoniae* were 2.14, 0.39, 0.38, and 2.5 mg/ml, respectively. The Minimum Bactericidal Concentrations (MBC) of silymarin were verified by the absence of bacterial growth of the isolated strains that were scattered from the lowest MIC. In conclusion, silymarin exhibited antibacterial efficacy against isolated *P. aeruginosa*, *S. aureus*, *E. coli*, and *M. ovipeumoniae* from pneumonic sheep *in vitro* compared to chloramphenicol, suggesting its therapeutic value in sheep.

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INTRODUCTION

Respiratory diseases are a prominent cause of financial loss in small ruminants, and they are associated with high mortality and poor conditions, making them a substantial impediment in the intensive production system (Abera and Mossie, 2023). The most prevalent microbiological pathogens are bacteria, viruses and fungi, with other risk factors such as high density, poor management and environmental conditions increased susceptibility to respiratory infections (Sah *et al.*, 2021).

Macrolides, quinolones, beta lactams, and florfenicol are routinely used to treat pneumonia (Peng *et al.*, 2014). Quinolones have a broad spectrum of action and greater penetration into lung tissue, making them popular antibiotics for pneumonia (DeDonder *et al.*, 2016). Antibiotic resistance is currently a major public health concern (Alshehri *et al.*, 2022). Mycoplasmas are inherently resistant to β-lactams and all antimicrobials that target the cell wall due to absence of the cell wall (Gautier-Bouchardon, 2018). While, other antimicrobials that are ineffective against mycoplasmas include trimethoprim,

sulfonamides, polymyxins, rifampicin, and first-generation quinolones (Chernova *et al.*, 2016), also Pseudomonas aeruginosa possesses inherent resistance against multiple types of antimicrobials (Wood *et al.*, 2023). *S. aureus* showed concerning levels of resistance to widely used antimicrobial drugs including tetracycline and penicillin (Grima *et al.*, 2021). Herbal medicine is becoming increasingly crucial in the fight against multidrug-resistant bacteria, as new generations of antimicrobials are ineffective (Beebe, 2023; Haghshenas *et al.*, 2023).

Milk thistle is a plant-derived compounds; silybin, the extract's principal bioactive component, is the key component of flavonolignans combinations (Holasová *et al.*, 2022). Silybin had significant antifungal and antibacterial efficacy against *E. coli* and potential synergistic properties when combined with antibacterial medicines (Rakelly de Oliveira *et al*., 2015). However, further studies are required to evaluate silymarin antibacterial activity in bacterial pneumonic sheep.

Milk thistle contains a complex mixture of flavonolignans called silymarin, which is the main bioactive ingredient produced and has various therapeutic benefits, such as antioxidant activity, and scavenging free radicals (Akhtar *et al.*, 2023), considered the most active extract, Silybum marianum extract under laser irradiation exhibits positive sensitivity for tested microorganisms (Aldayel, 2023). Silymarin possesses a wide range of biological and pharmacological actions via interacting with various inflammatory mediators, transcription factors, protein kinases (Wadhwa *et al.*, 2022).

The current study aimed to assess silymarin antibacterial efficacy against the isolated bacterial strains from pneumonic sheep in vitro as a primary study. We hypothesized that silymarin will have more antibacterial efficacy compared to chloramphenicol against isolated bacterial strains from pneumonic sheep.

MATERIALS AND METHODS

Animals examination and samples collection: A total of 75 sheep (1-2 years old) in Sadat City, Egypt, were physically examined during routine veterinary visits belonging a private farm from February 2022 to May 2022. Of the enrolled sheep, 25 were diagnosed as pneumonic exhibited respiratory symptoms including cough, nasal discharge with abnormal pulmonary sounds, depressive symptoms, and fever. Nasopharyngeal swabs were collected from sick sheep by strile swab after handling the sheep head in a standing posture and cleaning the nostrils with 70% alcohol then placed the sterile swab medioventrally in the nasal cavity and rotated the swab multiple times against the mucosa as described by (Garzon *et al.*, 2023). The collected swabs were immediately placed in nutrient broth for isolation of caustive bacterial species, and PPLO broth for Mycoplasma spp. All processed swabs were sent to the lab for bacterial examination in an ice box under cold conditions.

Phenotypic characterization of bacterial species isolated in this study: The isolation of *P. aeruginosa* was carried out by aerobic cultivation on Pseudomonas selective media and blood agars for 48 hours at 37°C. A bluish green color confirmed the distinct colonies. Baird-Parker (Oxoid Ltd., Basingstoke, UK) was used for *S. aureus* isolation at 37°C for 48 hours (Wehr and Frank, 2004).

For *E. coli* isolation, all samples were cultured at 37°C for 12 hours in nutritional broth (mTSB, Difco La Jolla, CA, USA), followed by 24 hours of culturing in a selective medium (MacConkey agar, MAC, Difco). Lactose fermenting colonies were then sub-cultured in Eosin methylene blue (medium (EMB; Difco). Metallic green sheen colonies were classified as *E. coli* (Cowan and Steel, 1974).

The processed samples were cultured in PPLO broth for three days at 37°C and transferred to PPLO agar medium to be examined every three days under a stereomicroscope. The agar blocks containing mycoplasma colonies were added to broth medium and cultured at 37°C for 3 days before purification. The digitonin sensitivity disc was used to identify the *mycoplasma species* (Freundt, 1973), while the arginine deamination and glucose fermentation tests were used to characterize the organism's biochemistry (Howard *et al.*, 1994).

Molecular identification of isolated strains: The GF-1 Tissue DNA Extraction Kit, vivantis, was used to extract the DNA from the bacterial strain (GF-TD-050). The PCR reaction was conducted in a 50 µl total volume, which included 200 ng DNA templates, 1 µl of 10 pmol/µl of each primer, 25 µl of 2x My Taq Red Mix, and 50 µl of sterile water. At 260/230 nm, a spectrophotometer was used to finally determine the extracted DNA's concentration. The thermal profile was completed in a gradient thermal cycler (S1000 Thermal cycler Bio-RAD, Hercules, CA, USA), as instructed by the kit instructions. Table 1 displayed the primer sequences and the annealing temperature. Following electrophoresis in 1.5% agarose gel, the PCR products were captured on UV film using a gel documentation system.

Table 1: Primer's sequence, PCR cycling conditions for molecular detection of *P. aeruginosa, S. aureus, E. coli,* and *Mycoplasma spp.*

		Strain	Primer sequence	Fragment size (bp)	Primary denaturation	One cycle	35-40 cycles	Reference
				Secondary denaturation	Annealing	Extension	Final extension	
P. aeruginosa (toxA)	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396 bp	94°C 5 minutes	94°C 1 minutes	55°C 60 second	72 °C 60 seconds	72 °C 7 minutes	(Matar *et al.*, 2002)
s. aureus (9nuc)	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	270 bp	94°C 5 minutes	94°C 30 seconds	55°C 30 seconds	72 °C 60 seconds	72 °C 10 minutes	(Louie *et al.*, 2002)
E. coli (eaeA)	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248bp	95°C 5 minutes	95°C 30 seconds	60 °C 30 seconds	72 °C 30 seconds	72 °C 4 minutes	(Bisi-Johnson *et al.*, 2011)
Mycoplasma spp	AGACTCCTACGGGAGGCAGCA ACTAGCGATTCCGACTTCATG	390bp	94°C 5 minutes	94°C 60 seconds	55°C 60 seconds	72°C 90 seconds	72°C 10 minutes	(Alberti *et al.*, 2006)

Milk thistle extract (silybum marianum): Silymarin powder was provided by Medical Union Pharmaceuticals (MUP) Company, Egypt. The Milk Thistle powder consists of silymarin 50% with a potency of 104.49%, with code number 0111304600 and control number 2021000569.

Preparation of stock solution and test solutions: To select the best option for usage against the isolated bacterial species, measure the minimum inhibitory concentration. A stock solution of silymarin was made by dissolving 1120 mg in 1 ml of their relevant solvents dimethyl sulfoxide (DMSO). Based on this concentration, the compounds were diluted to get a concentration of 560, 280, 140, and 70 mg/ml (test solution).

Determination of the antimicrobial efficacy of silymarin against isolated bacterial species

Antibacterial activity of silymarin: The well-diffusion technique was used to assess silymarin's antibacterial efficacy against (*P. aeruginosa*, *S. aureus*, and *E. coli*). The results were estimated using the diameter of the inhibition zone as defined by (Patel *et al.*, 2011) and compared to the inhibition zone of chloramphenicol.

Determination of minimum inhibitory concentrations (MICs) of silymarin: The minimum inhibitory concentration (MIC) of silymarin was determined against isolated *M. ovipeumoniae* and compared to the activity of chloramphenicol 30 µg (Hannan, 2000). while, the MIC for *P. aeruginosa*, *S. aureus* and *E.coli* was performed as described by (Shah, 2001), using micro broth dilution method. In briefly, 100 μl of BHI (Brain Heart Infusion, HiMedia) broth and 100 μl of silymarin stock solutions were added to each well of a 96-well microtiter plate. All wells received 10 μl of 0.5 Mc Farland standard turbidity adjusted bacterial suspensions added to them. Additionally, a control negative without bacterial solution was employed. The plates were then incubated for 24 hours at 37°C. Three duplicate assays were performed. Two to three microliters of each well's suspension were aseptically transferred to sterile (Brain Heart Infusion Agar, HiMedia) plates that corresponded to the dilutions the following day. MIC is the lowest dose of silymarin that totally prevents isolates' ability to proliferate bacteria, (Patel *et al.*, 2011).

Bacterial growth with different concentrations of silymarin: Bacterial growth was measured at 600 nm wave length (Gene 5 microplate reader, EXL808IU, USA), to test different concentrations of silymarin at 70, 140, 280 and 560 mg/ml.

Evaluation of Minimum Bactericidal Concentrations (MBC) of silymarin: After determination of the minimum concentrations of the silymarin that indicated by no bacterial growth, the dishes were inoculated on Tryptone soya agar under septic conditions for 24 h at 37°*C*. The bacterial growth was investigated at a concentration comparable to that of silymarin suspension. The concentration of silymarin suspension was reported as MBC since it did not result in any bacterial growth on the inoculated Tryptone soy agar (Abedon *et al.*, 2011).

Statistical analyses: Prevalence of different bacterial pathogens in pneumonic sheep was calculated by Fisher's exact test. Bacterial growth curve based on different concentrations of silymarin for each isolated strain was generated using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was considered at $P < 0.05$.

RESULTS

Prevalence of different bacterial pathogens in pneumonic sheep: Twenty-five (33.3%) out of 75 examined sheep showed pneumonia signs includes cough, nasal discharges (serous to mucopurulent), dullness, and lung sound (harsh sound) with systemic reactions in some cases. The results of bacteriological culturing of the collected nasopharyngeal swabs from the diseased sheep were carried out through a series of traditional isolation and biochemical tests. The findings showed that the most frequently identified bacterial isolates in this investigation were *P. aeruginosa*, *S. aureus*, *E. coli*, *and M. ovipeumoniae*, with 28%, 28%, 28%, and 16%, respectively. The results were illustrated in Table 2.

Table 2: Prevalence of different bacterial pathogens in pneumonic sheep

Total examined animals	% of pneumonic sheep		*P. aeruginosa*		*S. aureus*		*E. coli*		*M. ovipeumoniae*	
75	n	%	n	%	n	%	n	%	n	%
	25	33.3	7	28	7	28	7	28	4	16

% was estimated according to the total number of diseased animals (25).

Phenotypic identification of *P. aeruginosa, S. aureus, E. coli, and M. ovipeumonia* isolated from pneumonic sheep: *P. aeruginosa* was identified based on the characteristic colony shape (bluish- green color colonies) on selective agar medium, *S. aureus* identification was performed through morphological characters (black colonies with opacity zone) on Baird-Parker medium, *E-coli* in EMB agar identified and showed green metallic sheen) green metallic sheen of *E. coli* colonies on EMB medium, while *M. ovipeumoniae* identification was based on fried egg colonies on PPLO medium (Fig. 1 A-D).

Molecular identification of *P. aeruginosa, S. aureus, E. coli,* and *M. ovipeumonia*: Three isolates from each *P. aeruginosa, S. aureus, and E. coli* were randomly selected to confirm by molecular detection of specific genes *tox*A, *nuc*, and *eae*A, at 396, 270, and 248 bp, respectively. Also, two isolates of the identified *M. ovipeumonia* were explicitly selected to confirm its detection by PCR using a common unique 16S rRNA gene, which was amplified at 1000bp (Fig. 2A-D).

Antimicrobial efficacy of silymarin against isolated bacterial species

Antibacterial activity of silymarin: With respect to the diameter of the inhibitory zone, silymarin demonstrated antibacterial action diameter against *P. aeruginosa* at concentrations of 280 and 560 mg/ml (14 and 16 mm, respectively), but chloramphenicol had minimal impact (4 mm). On the other hand, chloramphenicol exhibited more antibacterial activity diameter against *S. aureus, E. coli* (22 and 24 mm, respectively), compared to different concentrations of silymarin 280 and 560 mg/ml (12, 16, 16, and 20 mm respectively) (Table 3).

Table 3: Inhibition zone diameter (mm) of silymarin *Vs* Chloramphenicol on *P. aeruginosa, S. aureus,* and *E. coli.*

Organism	Silymarin at different concentrations				Chloramphenicol
	560 mg/ml	280 mg/ml	140 mg/ml	70 mg/ml	30 µg/ml
P. aeruginosa	16	14	0	0	4
S. aureus	16	12	0	0	22
E. coli	20	16	0	0	24

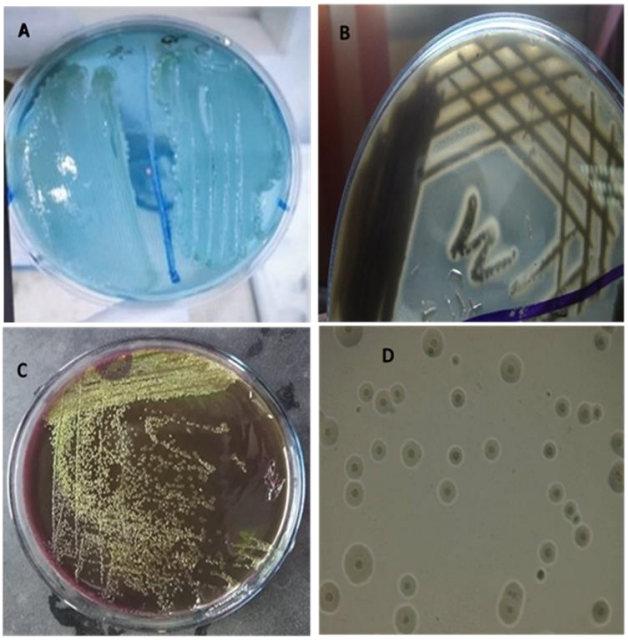

Fig. 1: Phenotypic identification of isolated bacterial strains, **(A):** bluish green color colonies of *P. aeruginosa* on Pseudomonas selective agar medium, **(B):** black colonies with opacity zone of *S.aureus* on Baird-Parker medium, **(C):** green metallic sheen of *E.coli* colonies on EMB medium, **(D):** characteristic colonies of *M. ovipeumonia* on PPLO medium (fried egg colonies) under stereomicroscope.

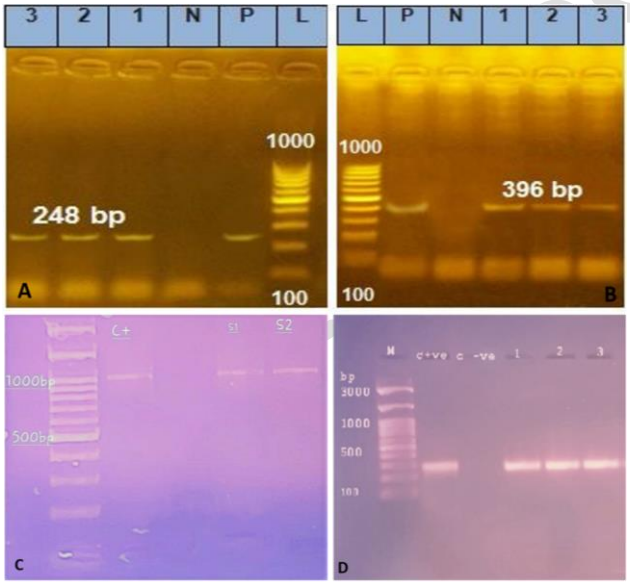

Fig. 2: Agarose gel electrophoresis (1.5%) of PCR product, **(A):** *eaeA* gene of *E. coli* at (248 bp). Lane: DNA Ladder. Positive samples from lane 1-3. N: control negative; P: control positive, **(B):** toxA of *P. aeruginosa* gene at (396 bp). Lane: DNA Ladder. Lane pos: positive control; Lane Neg: Negative. Positive samples from lane 1-3 positive samples, **(C):** 16 S rRNA at (1000 bp) for Mycoplasma. Lane: DNA Ladder. Lane C+: positive control; Positive samples (S1, S2), **(D):** of *nuc* gene at (270 bp) for *S.aureus,* Lane: DNA Ladder. Lane C+: positive control; Positive samples (1,2,3), +ve: control positive, -ve: control negative

Minimum inhibitory concentrations (MICs) of silymarin: The micro broth dilution technique was used to determine the minimum inhibitory concentration (MIC) of the silymarin against the isolated bacterial strains. The results showed that the MIC values for *P. aeruginosa, S. aureus, E. coli* and *M. ovipeumonia* were 2.14, 0.39, 0.38, and 2.5 mg/ml, respectively. As for Chloramphenicol, they were 64, 32, 32, and 0.5 mg/ml, respectively (Table 4). According to these findings, silymarin revealed antibacterial activity at 280 mg/mL with a MIC lower than chloramphenicol against *P. aeruginosa, S. aureus, E. coli*, and *M. ovipeumoniae*.

Bacterial growth with different concentrations of silymarin*:* Bacterial growth curve based on different concentrations of silymarin showed that *P. aeruginosa, S. aureus, E. coli, and M. ovipneumoniae* growth were significantly lower at concentrations of silymarin 280 mg/ml compared to other concentrations (Fig. 3).

Table 4: MIC concentrations of silymarin and chloramphenicol against isolated bacterial species from pneumonic sheep in vitro.

Organism	MIC of silymarin mg/ml at 280	MIC of chloramphenicol (30 µg/ml)
P. aeruginosa	2.14	64
S. aureus	0.39	32
E. coli	0.38	32
M. ovipeumonia	2.5	0.5

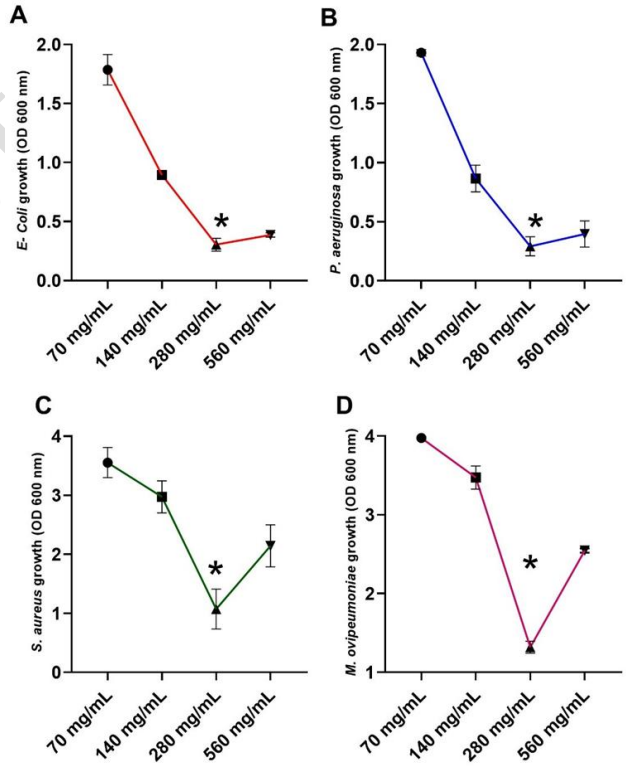

Fig. 3: Bacterial growth curve based on different concentrations of silymarin. **(A-D)** *E. coli, P. aeruginosa, S. aureus and M. ovipneumoniae* growth were significantly lower at concentrations of silymarin 280 mg/ml compared to other concentrations. (*P < 0.05).

Minimum Bactericidal Concentration (MBC) of silymarin: The MBC was verified by absence of bacterial growth of the examined strains scattered form the lowest MICs. Thus, confirmed that silymarin exhibited bactericidal activity against the isolated bacterial species.

DISCUSSION

P. aeruginosa has been implicated in various sheep infections such as respiratory disorders which are the major problems particularly pneumonia, attendant with physical and physiological stress, leading to major mortality rates and great economic loss (Bangar *et al.*, 2016). Moreover, *P. aeruginosa* infection may lead to urogenital, gastrointestinal, sinusitis, and osteomyelitis disorders (Rasooli *et al.*, 2018). Moreover, *P. aeruginosa* exhibited multiple resistance to several antibiotics such as amikacin, chloramphenicol, and gentamycin as well as having several virulence elements (Dapgh *et al.*, 2019; Liew *et al.*, 2019). *M. ovipneumoniae* was involved in pneumonia among sheep and goats and combated high antimicrobial resistance (Hayajneh *et al.*, 2024; Jaÿ *et al.*, 2020).

The current study recorded that the overall prevalence rate of respiratory infection in sheep was 33.3%, and the most frequently identified bacterial isolates in this investigation were *P. aeruginosa, S. aureus, E. coli, and M. ovipeumoniae*, were 28%, 28%, 28%, and 16%, respectively. In a Brazilian study, there was a nearly similar prevalence of 32.32% of 99 sheep diagnosed as pneumonic disorders (Franco *et al.*, 2019). Lower prevalence results were previously recorded in Germany through 12- month survey with a 20.9% (Radon and Winter, 2003). In a comparative study, a higher prevalence rate of 38.6% among sheep flocks reared in villages in the south area of Ethiopia (Ferede *et al.*, 2014). Moreover, the authors reported a variety of bacterial species were implicated in respiratory disease cases, including *bacillus* spp, *streptococcus* spp., and *staphylococcus* spp., as well as, *mollicutes* spp with no isolation of *pasteurella spp*.

In the current study, PCR was used efficiently for the detection of different bacterial species recorded in our study, (P. *aeruginosa, S. aureus,* and *E. coli*) using specific *tox*A, *nuc*, and *eae*A, at 396, 270, and 248 bp, respectively, as well as common universal 16S rRNA primer universal gene for *M. ovipeumonia* at 1000bp. In the same context, Dhama *et al.*, (2012) concluded that mycoplasma spp. identification is dependent on diverse primers. Moreover, a recent comparative study in Iraq confirmed that seven bacterial species were identified from pneumonic cases in small ruminants with a predominance of *S. aureus* and a lower prevalence of *pseudomonous* spp (Ahmed and Abdullah, 2022). Also, the authors successfully amplified the target genes in isolated bacterial species using species- specific genes, including *uid*A, and *nuc*, O-antigen acetylase gene targeting *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively.

Regarding the results of silymarin efficacy against the isolated bacterial species in the current study, silymarin had an antibacterial effect on *P. aeruginosa, S. aureus, E. coli,* and *M. ovipeumoniae*. Similar findings were described by (Evren and Yurtcu, 2015), who recorded the antimicrobial activity of silymarin between 60 and 120 mg/ml counter to gram-positive bacteria, and silymarin also had antibiofilm activity when added as a dietary supplement. This is agreed with a study in Iraq that revealed that silymarin had antibacterial effect against *P. aeruginosa, E. coli*, and *A. baumannii, S. aureus, MRSA, E. faecalis* as well as antifungal activity against *C. glabrata, C. albicans* and *C. krusei* (Mohammed *et al.*, 2019). In the same line, (Abdelazim, 2017) recorded the antimicrobial activities of different silymarin concentrations against *B. subtilis*, *B. cereus* and *S. aureus*, *E. coli*, and *P. aeruginosa*, molds as *A. niger*, *A. flavus*, *A. parasiticus*, Penicillium sp. and yeast as *G. candidum*. Furthermore, silymarin had potent antibacterial action, particularly against gram- positive bacteria, including MRSA and some Streptococcus strains, through inhibition of protein synthesis (Lahlah *et al.*, 2012; Lee *et al.*, 2003). In our study, silymarin showed effective antibacterial against *M. ovipeumoniae and P. aeruginosa,* which classified as highly resistant pathogens to a variety of antibiotics. Silymarin had antibacterial activity through creating complexes with extracellular soluble proteins that attach to bacterial cell wall or counteract the cell membrane permeability, facilitating its invasion (Burt, 2004).

Conclusions: Silymarin, at a dosage of 280 mg/ ml had a minimum inhibitory concentration (MIC) against *P. aeruginosa, S. aureus, E. coli*, and *M. ovipeumoniae* that were isolated from pneumonic sheep, with special concern that *P. aeruginosa* and *M. ovipeumoniae* were resistant to chloramphenicol. In addition to the challenging orbiting of raw, pure silymarin material, and the examination of pneumonic sheep yielded just four distinct bacterial species, further information on silymarin's antibacterial ability against various bacterial species *in vitro*, and as an alternative natural therapeutic agent *in vivo*, further studies are required.

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Ethical approval: The current study was carried out according to the guidelines, regulations and ethical approval of the Faculty of Veterinary Medicine (Local ethical approval), University of Sadat City, Egypt (Approval no. VUSC-028-1-22).

Conflicts of interest: The authors declare that they have no conflicts of interest.

Author contribution: HH designed and follow up the study progress; AK, WM, AE, HK, AE, and AA have contributed equally to this work and share first authorship in the experiments; RT revised the manuscript for publication. All authors agreed the final manuscript.

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