

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

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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 antibiotic 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 inhibitory 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.


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/remarked the distinct colonies. Bairdus et al. (2021). Silybi...54x103]E. coli (eaeA) (toxA) P. aeruginosa Table 1: by strile swab after Nasopharyngeal sw sounds, depressive symptoms, and fever. cough, nasal discharge with abnormal pulmonary 2022. Of the enrolled sheep, 25 were diagnosed as belonging a private farm from February 2022 to May 2 years old) in Sadat City, Egypt, were physically examined during routine veterinary visits of 75 sheep (1 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 activity against the isolated bacterial strains from pneumonic sheep. Milk thistle contains a complex mixture of flavonolignans called silymarin, which is the main 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 activity 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 to a private farm from February 2022 to May 2022. Of the enrolled sheep, 25 were diagnosed as pneumonic exhibiting respiratory symptoms including cough, nasal discharge with abnormal pulmonary sounds, depressive symptoms, and fever. Nasopharyngeal swabs were collected from sick sheep by sterile 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>
<thead>
<tr>
<th>Strain</th>
<th>Primer sequence</th>
<th>Fragment size (bp)</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa (eaeA)</td>
<td>GACAAGCCTCAGCATCACGACG</td>
<td>396 bp</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>CGTGGCCCATTCGCTCCAGCGCT</td>
<td>270 bp</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td>s. aureus (9nu)</td>
<td>AGCGAGCCCTGACGCTTACGCT</td>
<td>390 bp</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td>E. coli (eaeA)</td>
<td>ATG CTT AGT GGT GTT TTA GG</td>
<td>248 bp</td>
<td>95°C</td>
<td>95°C</td>
<td>60°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td>Mycoplasma spp</td>
<td>AGACTCTCATGCGAGGCAAGGCA</td>
<td>390bp</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>ACTAGCGATTCGCGACTTATG</td>
<td>390 bp</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
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</table>
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. ovipneumoniae 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, EXL408IU, 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 pneumatic 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 pneumatic 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. ovipneumoniae, with 28%, 28%, 28%, and 16%, respectively. The results were illustrated in Table 2.

Phenotypic identification of P. aeruginosa, S. aureus, E. coli, and M. ovipneumonia isolated from pneumatic 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 EM agar identified and showed green metallic sheen) green metallic sheen of E. coli colonies on EMB medium, while M. ovipneumoniae 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. ovipneumonia: Three isolates from each P. aeruginosa, S. aureus, and E. coli were randomly selected to confirm by molecular detection of specific genes toxA, nuc, and eaeA, at 396, 270, and 248 bp, respectively. Also, two isolates of the identified M. ovipneumoniae 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.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Silymarin at different concentrations</th>
<th>Chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>560 mg/ml</td>
<td>280 mg/ml</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>S. aureus</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>E. coli</td>
<td>20</td>
<td>16</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC of silymarin mg/ml</th>
<th>MIC of chloramphenicol at 30 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>2.14</td>
<td>64</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.39</td>
<td>32</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.38</td>
<td>32</td>
</tr>
<tr>
<td>M. ovipneumonia</td>
<td>2.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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. ovipneumonia 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): 16S 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.

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 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. ovipneumoniae 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. ovipneumoniae.

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).

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 (Rasooli et al., 2019). Lower Moreover, (Abdelazim, 2003) (Evren et al., 2012) – here was a prevalence of pneumonia among sheep and goats and combated high antimicrobial resistance (Hayajneh et al., 2024; Jaf et al., 2020).

The current study reported 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. ovipneumoniae, 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 toxA, nuc, and eaeA, at 396, 270, and 248 bp, respectively, as well as common universal 16S rRNA primer universal gene for M. ovipneumonia at 1000bp. In the same context, Dham 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 uidA, and nuc, O-antigen acetylace 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. ovipneumoniae. 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. baumanii, 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. ovipneumoniae 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. ovipneumoniae that were isolated from pneumonic sheep, with special concern that P. aeruginosa and M. ovipneumoniae 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.

REFERENCES


