INHIBITORY EFFECT OF NEEM (*Azadirachta indica*) PLANT EXTRACT ON BOVINE AND POULTRY PATHOGENS FROM SRI LANKA

R. A. N. Karunathilaka [1,2*], N. P. S. M. Arachchige [1,3], G. A. Gunawardana[1] and G. A. Deepal Chandana[4]

[1]Veterinary Research Institute, Sri Lanka
[2]Faculty of Technology, University of Jaffna, Sri Lanka [3]Faculty of Agriculture, University of Peradeniya, Sri Lanka [4]Veterinary Investigation Center, Sri Lanka

Over several decades, microbes have developed resistance to antimicrobial agents in varying degrees. WHO has identified antimicrobial resistance as a serious threat to global health and food security. Antibiotic resistance among animal pathogens has increasingly reported in Sri Lanka as well. Hence, the need for alternatives to deplete pathogens is imperative. One effective approach is herbal extracts with medicinal properties. This study aimed to evaluate the antimicrobial effects of Neem (*Azadirachta indica*) plant extract on bovine and poultry pathogens. Seventeen bacterial strains comprising 11 bovine mastitis isolates (*E. coli* = 5, Staphylococcus = 3, other Coliforms = 3) and six poultry Salmonella isolates were tested. From 12g of fresh Neem (Kohomba) leaves, aqueous leaf extract was obtained after crushing. Paper disks containing 250 µl of the Neem extract were used in paper disk diffusion method to assess the bacterial inhibition on Muller–Hinton agar, as described previously. Statistical analysis was performed using MedCalc (Version 22.023). Neem extract inhibited 12 isolates, including seven bovine pathogens and five poultry Salmonella strains. Especially, Neem inhibited the Staphylococcus isolates with zone diameters of 27mm, 23mm and 20mm. The three Coliforms showed inhibition zone diameters of 25mm, 23mm and 22mm. Among the five *E. coli* strains, one showed the largest inhibition zone, 32mm while other four were not inhibited. Neem demonstrated a higher inhibition zone of 23mm against one Salmonella strain whereas the remaining four were of 9mm. One Salmonella was not inhibited. Among inhibited isolates, mean inhibition-zone value of bovine pathogens was significantly higher (P = 0.001) than poultry Salmonella isolates. Antibacterial activity of Neem against Staphylococci and Coliforms confirms its suitability as a herbal approach to reduce mastitis. Similarly, Neem supplements can be used to enhance poultry health by suppressing Salmonella. Unaffected *E. coli* and Salmonella may possess extra virulent components. However, prolonged exposure to a higher quantity of Neem would be bactericidal to those. Current study highlights the potential use of Neem to minimize antibiotic usage in animal husbandry. Further research is in progress to develop an eco-friendly Neem product beneficial for livestock farmers.

Keywords: *Azadirachta indica*, mastitis, Neem, poultry Salmonella

[*]*Corresponding Author: ayeshanimanthiinfo99@gmail.com*

INHIBITORY EFFECT OF NEEM (*Azadirachta indica*) PLANT EXTRACT ON BOVINE AND POULTRY PATHOGENS FROM SRI LANKA

R. A. N. Karunathilaka [1,2*], N. P. S. M. Arachchige [1,3], G. A. Gunawardana[1] and G. A. Deepal Chandana[4]

[1]Veterinary Research Institute, Sri Lanka
[2]Faculty of Technology, University of Jaffna, Sri Lanka
[3]Faculty of Agriculture, University of Peradeniya, Sri Lanka
[4]Veterinary Investigation Center, Sri Lanka

INTRODUCTION

Antimicrobial resistance (AMR) occurs when microorganisms develop the ability to evade the killing or growth inhibitory effects of antimicrobials against those organisms. In the past few decades, misuse and overuse of antibiotics have led to antibiotic resistance among bacteria (Singer *et al.*, 2003). This has a direct impact on human and animal health, food safety and food security. WHO has listed AMR as one of the top global health crises. Antibiotic resistance among animal pathogens has increasingly reported in Sri Lanka as well. To overcome the AMR problem, attention has been drawn toward the environmentally friendly, sustainable approaches. Plant extracts are potential alternatives to antimicrobials, to deplete pathogens. As an ecofriendly solution, Neem (*Azadirachta indica)* can be used to reduce the antibiotic usage since Neem contains various types of secondary metabolites such as azadirachtin, flavonoids, tannins and nimbidin which are detrimental to microbes. Bovine mastitis is a serious threat to dairy cattle (Sungkatavat *et al.*, 2023). Due to mastitis, the milk yield, quality and dairy health declines immediately. Similarly, Salmonellosis has been a main poultry disease of economic and public health importance. There is a high potential to use Neem for minimizing bovine mastitis and poultry *Salmonella spp.* This study focuses on evaluating the inhibitory effect of Neem (Kohomba) plant extract on bovine and poultry pathogens obtained from local animal farms for the purpose of reducing the antibiotic usage.

METHODOLOGY

Preparation of Neem plant leave extract

Fresh Neem (*Azadirachta indica*) leaves were collected and 12g was weighed using analytical balance and washed with distilled water to remove any surface contamination. Then, the washed leaves were air-dried in a sterile environment at room temperature. The 12g of leaves were aseptically cut into small pieces by using sterile knife and a sterile cutting-board. The cut leaves were further crushed by using a sterile motor and pestle under aseptic conditions to obtain aqueous leaf extract. This step is performed for the purpose of releasing the bioactive compounds which contain in Neem plant leaves. The ground leaf material was manually extracted to obtain the Neem extract. Throughout the extraction process, sterile gloves were used to prevent contamination. Finally, 20ml volume of Neem plant leave extract was added into sterilized universal bottles.

Neem paper disks preparation

Filter paper disks were prepared with the weight of 0.03g. From the 20ml of Neem plant leave extract, 250µl of volume was pipetted out. Then, the volume was pressed into 0.03g of filter paper disks. Each and every paper disks were treated with Neem extract by using same volume of 250 µl. Then, prepared filter paper disks were kept in incubator at 37^0C for 5-10 min for drying.

Mastitis and poultry pathogen samples preparation

In this study, Disk diffusion method (Zaidan *et al.*, 2005) was performed using Neem plant extract to evaluate the inhibition zones against 17 bacterial pathogens on Muller – Hinton agar surface. The

tested pathogens included bovine bacteria (n =11) obtained from mastitis cases belonged to *Staphylococcus spp* (n = 3), *Coliform spp* (n = 3) *E. coli* strains (n = 5) and poultry *Salmonella spp* (n = 6) from clinical cases. The pathogen inoculating suspensions were prepared by dissolving bacterial colonies in 9ml of Normal Saline and adjusting the cell turbidity equal to McFarland standard 0.5. Then, the suspensions were plated on Muller-Hinton agar using a sterile cotton swab. After inoculating the targeted microbes, the Neem extract containing paper disks were placed in the desired points on the agar surface and plates were incubated at 37^0C for overnight. The inhibition zones were measured subsequently. Disks with Normal Saline was used as a control. Results were analyzed statistically using Medcalc (Version 22.023).

RESULTS AND DISCUSSION

In this experiment seventeen bacterial strains comprising 11 bovine mastitis isolates *Staphylococcus spp* = 3, other *Coliforms spp* = 3, *E. coli* = 5) and six poultry *Salmonella spp* were tested. A total of 12 pathogens were inhibited by Neem extract. Seven bovine and five poultry pathogens were sensitive to Neem. Resulted inhibition zones with Neem extract is shown in figure 1.

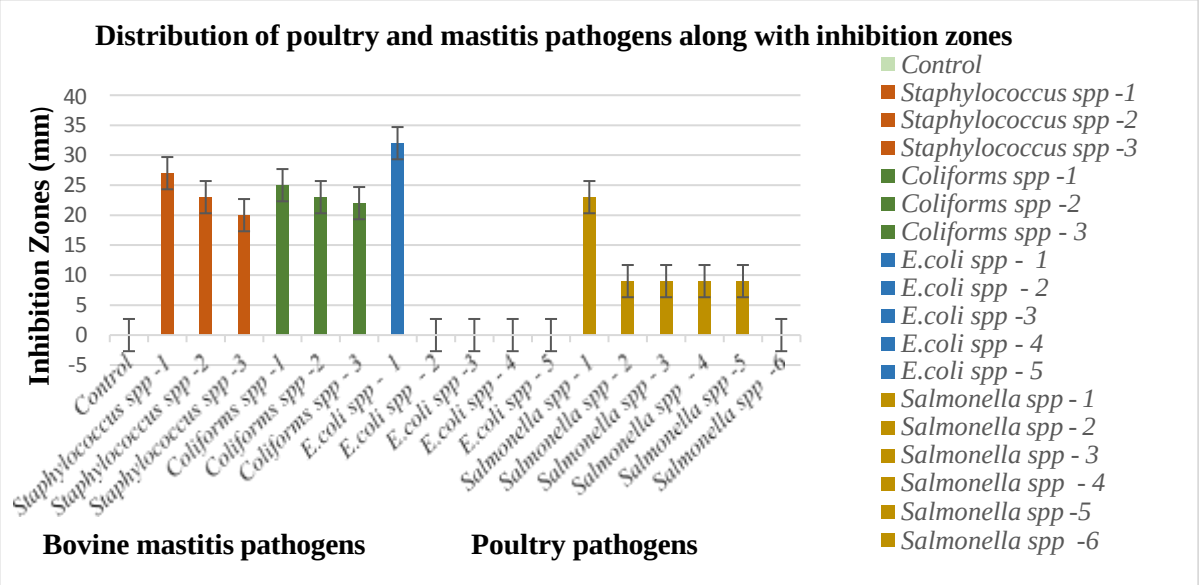

Figure 1: Distribution of poultry and mastitis pathogens along with diameter of inhibition zones

Results of mastitis pathogens

Seven bovine pathogens displayed inhibition zones. Figure 2 indicates the inhibition zones produced against a bacterial strain of *Staphylococcus.*

Figure 1: Inhibition zone against Staphylococcus spp of mastitis pathogens

Out of three isolates, Neem inhibited the three *staphylococcus* isolates with zone diameters of 27mm, 23mm and 20mm. Various virulence factors of *Staphylococcus spp* exhibited plethora of toxins, immune evasion factors and proteins and non-protein factors have caused to arise mastitis in dairy cattle (Cheung *et al.,* 2021). Results of inhibition zones revealed that Neem (Kohomba) has the ability to oppose such virulence factors of *Staphylococcus spp*. This is a valuable discovery in the farm sector for enhancing the animal health and welfare. *Staphylococcus aureus* shown adverse effects on antibiotic resistance. So, Neem can be used as a sustainable solution to solve the problems of bovine mastitis.

When consider the mastitis causing *Coliforms spp,* all three isolates showed inhibition zones with diameters of 25mm, 23mm and 22mm. Mastitis causing *Coliform spp* can possess especial characters of resistance to the serum bactericidal activity and evading phagocytosis (Hogan *et al.,* 2003). According to the results, Neem plant extract showed antimicrobial properties against tested *Coliform spp.* Due to that reason, inhibition zones of considerable sizes of diameters were produced.

Among the five *E. Coli* strains, one showed the largest inhibition zone, 32mm while other four were not inhibited. There are various virulent factors shown by *E. coli* strains such as adhesion molecules, iron uptake systems and invasion proteins and toxins. This 32mm inhibition zone showed that the Neem has a great influence on suppressing the mechanism of pathogenic *E. Coli*. Also, there were four isolates not showing any inhibition zones indicating certain virulence factors of *E. coli* strains were varying from each other. Also, the bioactive compounds concentration of the leaf extract may be too low to work against these four *E. coli* strains. These strains may be having higher virulence. If the Neem extract volume 250µl is increased for these four *E. coli* strains, there may be inhibition zones produced.

Results of *Salmonella spp* - Poultry pathogens

Salmonella spp of poultry pathogens in farms causes, poor general conditions (such as ruffled feathers, pale and small crest), diarrhea, lower feed intake and lower egg production with high mortality. This is a serious issue since the health and productivity of poultry will be diminished (Nirmala *et al.,* 2018). In this investigation, Neem extract demonstrated a higher inhibition zone of 23mm against one *salmonella* strain whereas the remaining four showed zones less than 10mm. One *salmonella* was not inhibited. The 23mm inhibition zone indicated that Neem was able to destroy certain virulence characters of Salmonella including plasmids, toxins and flagella. Hence, Neem can be used in farms effectively as a sustainable solution. Out of six poultry pathogen samples, four poultry pathogens exhibited smaller size (less than 10mm) of inhibition zones. This may be a reason; the production of toxins and virulent components may be higher in these four isolates when compared to the previously inhibited *Salmonella spp.* One strain of Salmonella was not inhibited and this strain may be of high pathogenicity.
Results of statistical tests are shown in Table 1 and Table 2.

Table 1: Comparison of mean inhibition zone values (mm) of bovine pathogens and poultry pathogens after exposure to Neem

Sample 01 – Bovine pathogens	
Mean	24.57
Standard deviation	3.95
Sample size	7
Sample 02 – Poultry pathogens	
Mean	11.8
Standard deviation	6.26
Sample size	5

Table 2: Statistical significance between inhibition zones demonstrated by bovine and poultry pathogens with the effect of Neem

Difference	-12.770
Standard error	2.930
Significance level	P = 0.0014

A statistically significant higher inhibition zone was observed for bovine pathogens (n = 7) (P = < 0.05) than poultry pathogens (n = 5). The significance level was P = 0.0014. Neem was more effective in inhibiting the tested three groups of mastitis causing pathogens. By considering the poultry pathogen inhibition zone variations, it would be able to optimize the concentration of Neem extract and exposure time for getting higher effectiveness to inhibit the poultry pathogens. According to that, there is a huge potential to develop a farmer friendly green sprayer using Neem.

In this study, a large diameter (more than 20mm) of inhibition zones indicated that Neem can be used successfully for suppressing the growth of bovine and poultry pathogens. Small diameter (less than 10mm) of inhibition zones indicated the higher pathogenicity. The results of this experiment revealed that Neem can be used effectively against bovine and poultry pathogens.

CONCLUSIONS

In conclusion, the current study highlights the perceived effectiveness of Neem in preventing livestock pathogens that cause mastitis and poultry *Salmonella*. Hence, this can contribute for minimizing the antibiotics usage and antimicrobial resistance (AMR) within farm animal population. Further research is in progress to develop an environmentally friendly green sprayer from Neem extract beneficial for livestock farmers.

ACKNOWLEDGMENT

This study was supported by the Department of Animal Production and Health, Peradeniya for funding.

REFERENCES

Cheung, G. Y., Bae, J. S., & otto, M. (2021). Pathogenicity and virulence of staphylococcus aureus. Virulence, 12(1), 547-569.

Hogan, J. and Smith, K.L., 2003. Coliform mastitis. Veterinary research, 34 (5), pp.507-519.

MedCalc Software Ltd. Comparison of means calculator. https://www.medcalc.or/calc/comparison_of_means.php (Version 22.023)

Nirmala, T.V., Reddy, A.D., Karuna Sree, E., Venkata Subbaiah, K. and Shali Raju, G., 2018. Salmonellosis in poultry: A case report. *International journal of Current Microbiology and Applied Sciences,* 7 (2), pp.2347 -2349.

Singer, R. S., Finch, R., Wegener, H. C., Bywater, R., Walters, J., & Lipsitch, M. (2003). Antibiotic resistance—the interplay between antibiotic use in animals and human beings. The lancet infectious diseases, 3(1), 47-51.

Sungkatavat, P., Khongkhai, H., Kanchana, W., Saengsawarng, P., Sangkanu, S., Nissapatorn, V., & Mitsuwan, W. (2023). Piper betle extract and its application in bovine teat dipping solution inhibit and eliminate biofilms in bovine mastitis-inducing staphylococci. Veterinary world, 16(10), 2135. Zaidan, M.R., Noor Rain, A., Badrul, A.R., Adlin, A., Norazah, A., & Zakiah, I. (2005). In vitro screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Trop biomed,* 22 (2), 165 – 170.