
EXVIVO STUDIES ON PHARMACODYNAMIC INTERACTION OF PHYTOCHEMICALS WITH ANTIBIOTICS AGAINST CLINICAL ISOLATES FROM MASTITIC MILK SAMPLES

G. Srividya¹, B. Deepthi ², S.Lakshminarasaiah², G Srinivasarao¹

¹Department of Veterinary Pharmacology & Toxicology

²Department of Veterinary Microbiology

ABSTRACT

Drug interaction is the effect produced by combination of various drugs administered in a biological system which may produce beneficial or adverse effects. Due to continuous usage of antibiotics to bacterial infections, pathogenic bacteria developed resistance mechanisms which leads to reduced antibacterial activity of different antibiotics. Combination of antibiotics with phytochemicals became an alternative to enhance the efficacy of antibiotics. In this connection, in the present study, the effect of certain phytochemicals namely Quercetin, Morin, Ellagic acid, Chlorogenic acid, Rutin and Naringenin in combination with antibiotics Amoxycillin and Ciprofloxacin were evaluated. *Staphylococcus aureus* and *Escherichia coli* were used as test organisms. They were isolated from the mastitis milk samples and their presence was confirmed using Polymerase Chain Reaction. The antibacterial activity of the antibiotics and antibiotics in combination with phytochemicals were determined using broth dilution method and agar well diffusion assay. The MIC and zones of inhibition were used as indicators for drug interaction.

Key words: Drug interactions, Phytochemicals, *E.coli*, *Staphylococcus aureus*

INTRODUCTION

Mastitis is one of the common disease observed in livestock which causes production as well as economic loss to the farming community. Among the several causative agents of mastitis, the major bacterial species responsible are *Escherichia coli* and *Staphylococcus aureus*. These two bacteria are generally more prone for antibiotic resistance due to various reasons. Antibiotic resistance is the major drawback responsible for therapeutic failure in mastitis caused by the bacteria

such as *E.coli* and *Staphylococcus aureus*. Even the selective antibiotics are available for the above bacteria commonly observed in mastitis cases the success of the treatment is not upto the mark till today.

Phytochemicals act synergistically with antibiotics, reduce the development of resistance to antibiotics and improve the efficacy of antibiotics (Savoia ;2012). Phytochemicals are the compounds present in plants also called as natural drugs which possess various pharmacological activities such as antineoplastic, antioxidant

, antibacterial, antiviral, anti-inflammatory and immunomodulatory activities. Natural compounds such as Oregano, Carvacrol and Thymol showed antibacterial activity by inhibiting the biofilm formation of *Staphylococcus aureus* and *Staphylococcus epidermidis* invitro. (Nostro; etal 2007). Similarly, ascorbic acid influences the Ciprorofloxacin MIC against *Escherichia coli* invitro (Srividya etal; 2017). As the natural compounds altering the antibacterial activity of phytochemicals, In the present study we determined the antibacterial activity of Amoxicillin and Ciprofloxacin against *Staphylococcus aureus* and *Escherichia coli* alone as well as in combination with selected phytochemicals Quercetin, Morin, Ellagic acid, Chlorogenic acid, Rutin and Naringenin.

MATERIALS AND METHODS

Drugs and chemicals

Amoxicillin (Neovet), Ciprofloxacin, DMSO, Mueller Hilton broth (MHB), Mueller Hilton agar (MHA), Trypticsoya broth (TSB) were procured from Himedia. Quercetin, Morin, Rutin, Ellagic acid and Chlorogenic acid were procured from Sigma chemicals. All the phytochemicals were dissolved in DMSO for preparing the stock solutions of 10mg/ml.

Isolation of organism

Milk samples were collected from buffaloes suffering from clinical mastitis. The milk samples were inoculated into TSB and incubated at 37°C for 18hr. After incubation the inoculum was streaked on

MacConkey Agar, a differential media for *E. coli*. A single colony was then picked and streaked on Nutrient agar slant. The cultural characteristics of isolates were confirmed by streaking the pure culture on Eosin Methylene Blue Agar (EMB). The EMB agar showed greenish metallic sheen with black centered colonies which indicates *E. coli*. The inoculums obtained from milk samples incubated in TSB at 37°C for 18 hours were streaked on Mannitol Salt Agar for the isolation of *S. aureus*. The *Staphylococcus aureus* showed yellow coloured colonies on MSA.

Identification of isolated bacteria by PCR

Isolation of bacterial DNA : The 18hr culture was inoculated in TSB and incubated at 37 °C for 18 hrs. After incubation 2ml of the bacterial culture was centrifuged at 5000rpm for 10 min. The pellet of bacterial cell mass was collected. The DNA template from bacterial cell mass was isolated by high salt method of DNA extraction (Aravindakshan et al; 1977) with suitable modifications.

The DNA template isolated was confirmed with primers Eco 2083 GCT TGA CAC TCA ACA TTG AG AND Eco 2745 GCA CTT ATC TCT TCC GCA TT (Riffon etal 2001).

The DNA template isolated was confirmed with primers Staur4 ACG GAG TTA CAA AG G ACG AC and Staur6 AGC TCA GCC TTA ACG AGT AC (Straub etal, 1999)

Evaluation of antibacterial activity

Micro broth dilution method

A sterile 96 well flat bottomed plastic tissue culture plate with a lid was taken and filled 100 μ l of MHB to all wells. 100 μ l of the test compound was added to the first well and serially diluted. The 18hr bacterial culture was taken and adjusted to 0.5 Mc Farland standard. 10 μ l of the bacterial culture was added uniformly to all wells and incubated at 37^o C for 18- 24 hrs. The absorbance was taken at 660nm and the bacterial growth was confirmed by development of pink colour with the addition of *p*- Iodo NitroTetrazolium (INT). The minimum concentration of the test compound where it inhibits the bacterial growth is recorded and taken as minimum inhibitory concentration (MIC). (NCCLS 2000).

Agar well diffusion assay

The antibacterial activity of the selected phytochemicals was determined by agar well diffusion assay. 3.8 gm of MHA was dissolved in 100ml of distilled water and autoclaved at 121^oC for 15 min. It was allowed to cool and then poured in petriplates. After solidification the plates were inoculated with 18hr culture by sterile cotton swabs, then wells were created using a sterile cork borer and these were sealed properly with 1% agarose to avoid seepage of the test compound. 10 μ l of the test compound was added to the wells and plates were incubated at 37^oC for 12 – 24 hr. Areas of zone of inhibition was measured in millimetres using the formula πr^2

Statistical analysis

The differences of MIC values and zones of inhibition in agarose diffusion assays among various groups was tested using ANOVA as implemented in SPSS V17.

RESULTS

The mastitic milk samples were cultured in TSB. Genomic DNA was isolated from the culture to confirm the samples for the presence of *S. aureus* and *E. coli* using PCR technique (Figures 1 and 2). The positive samples of *S. aureus* and *E. coli* were further grown on specific media to isolate the specific organism. Thus isolated *S. aureus* and *E. coli* cultures were further used for microbroth dilution technique and ABST to understand the efficacy of antibiotic and natural compound synergistic activity. The pure antibiotic was used as control. The microbroth dilution technique indicated that Quercetin is the most efficient phytochemical against *S. aureus* among the tested natural compounds which is comparable with Amoxycillin (Table 1). Similarly, the pure form of Ciprofloxacin is most effective against *E. coli* and Morin is the most efficient phytochemical against *E. coli* among the natural compounds tested (Table 1).

The Amoxycillin and Quercetin combination was tested for ABST using *S. aureus* culture to understand the synergistic effect of the antibiotic and phytochemical antibiotic combination. When Quercetin alone was used, the zone of inhibition was very low. When Amoxycillin Quercetin combination was used, the zone of

inhibition was improved significantly and is comparable to the zone of inhibition of Amoxycillin alone used. (Table 2).

Based on the results of micro broth dilution technique, the combination of Ciporfloxacin and Morin was used to test the efficiency of antibiotic and phytochemical combination. In addition, the combination of Ciprofloxacin and Quercetin was also tested to understand the synergistic effect of the antibiotic and phytochemical combination. The combination of Ciprofloxacin and Morin was observed to be less efficient compared to Ciprofloxacin alone or Ciprofloxacin in combination with Quercetin (Table 3).

DISCUSSION

Antibiotics combined with natural compounds is of increasing interest in research to combat emergence of drug resistance. Application of the natural compounds for the treatment of ailments is an ancient practice. However, the natural compounds suffer from the disadvantage that their activity is weak in general and often non-specific (Srivastava et al., 2014). The modern antibiotics often suffer from development of resistance to microorganisms. Hence of late, a combination of phytochemicals and antimicrobials appears to be an effective method of dealing with antimicrobial resistance of antibiotics (Inui et al., 2007). Phytochemicals as such may not possess the significant antibacterial activity but application of these phytochemicals along with antimicrobial agents alters the pharmacological properties of antibiotic. (Z de Sousa Silveria et al., 2017)

In the present study we determined the antibacterial activity of phytochemicals, antibiotics alone as well as in combination by broth dilution method and Agar well diffusion assay. Quercetin showed lowest minimum inhibitory concentration compared to Amoxycillin against *S. aureus*, though the difference is not statistically significant. But agarose diffusion assay showed poor antibacterial activity of Quercetin alone against *S. aureus*. However, when Quercetin used in combination with Amoxycillin, due to synergistic effect, the combination produced strong antimicrobial activity that is comparable to the use of antibiotic alone at higher dose (Table 2). The results are in agreement with previous studies indicating synergistic effect of Quercetin in combination with antibiotics (Kyaw et al 2012). Similarly combination of Quercetin and Morin with Ciprofloxacin also produced similar effect of usage of Ciprofloxacin alone even at lower doses (Table 3).

The present study suggests that usage of Quercetin in combination with antibiotics like Amoxycillin and Ciprofloxacin at lower doses can produce similar effect of the Antibiotic at higher dose. Such combination therapies may help in reducing resistance to antibiotics in mastitis therapy.

REFERENCES

- Aravindakshan TV, Nainar AM and NachimuthuK , High salt method : A simple and rapid procedure for isolation of genomic DNA from buffalo white blood cells (1977). Indian Journal of Experimental Biology, Aug 35 (8) : 903- 5.

- Inui T, Wang Y, Deng S, Smith DC, Franzblau SG, Pauli GF.(2007) Counter-current chromatography based analysis of synergy in an anti-tuberculosis ethnobotanical. J Chromatogr A.; 1151:211–5.
- Kyaw B.M, Shuchi arora, and Chu Sing Lim.(2012)Bactericidal antibiotic-phytochemical combinations against methicillin resistant *Staphylococcus aureus*Braz J Microbiol. Jul-Sep; 43(3): 938–945
- NCCLS - National Committee for Clinical Laboratory Standards (2000), Approved standard M7-A5. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 5. ed. NCCLS, Wayne
- Riffon, R.; Sayasaith, K.; Khalil, H.; Dubreuil, P.; Drolet, M.; Lagacé, J.(2001) Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. J. Clin. Microbiology, **39** (7) : 2584-2589.
- Savoia D (2012)Plant-derived antimicrobial compounds: alternatives to antibiotics Future Microbiology, Aug;7(8):979-90.
- Srivastava J, Chandra H, Nautiyal AR, Kalra SJ. (2014)Antimicrobial resistance (AMR) and 5 plant-derived antimicrobials (PDAMs) as an alternative drug line to control infections. 3 Biotech. ;4:451–60.
- SrividyaG, Deepthi B, S.Lakshminarasaiah (2017) Ascorbic acid enhances Ciprofloxacin antibacterial activity against isolates of *Escherichia. coli* from subclinical mastitis cases of buffaloes.International journal of Veterinary Sciences and Animal Husbandry.2(5):21-24.
- Straub, J. A., Hertel, C. and Hammes, W.P.(1999). A 23S rRNA targeted polymerase chain reaction-based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy products. Journal of Food Protection, 62: 1150–1156.
- Z de Sousa Silveira, Z.,Macêdo, N.S., de Freitas, T.S.,da Silva, A.R.P.,dos Santos, J.F.S.,Morais-Braga, M.F.B.,da Costa, J.G.M.,Teixeira, R.N.P.,Kamdem, J.P.,Coutinho, H.D.M.,da Cuda Cunha, F.A.B.(2017) Antibacterial enhancement of antibiotic activity by *Enterolobium contortisiliquum* (Vell.) Morong Asian Pacific Journal of Tropical Biomedicine, Vol 7,(10),: 945-949

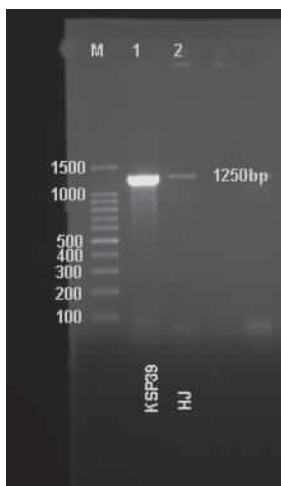


Figure 1 PCR amplification of *S. aureus*
Lane M -100bp ladder,Lane 1- *S.aureus*
isolate ,Lane2-*S.aureus* isolate



Figure 2. PCR amplification of *E. coli*
Lane M -100bp ladder,Lane 1- *E.coli*
isolate ,Lane2-*E.coli* isolate

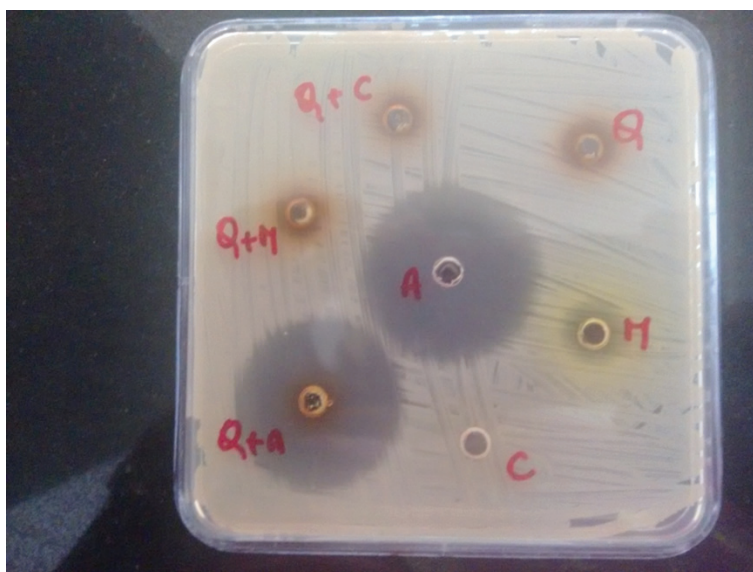


Figure 3. *Staphylococcus aureus* Agar well diffusion assay (C - Control, Q - Quercitin, M -Morin, A - Amoxycillin, Q+A - Quercitin+Amoxycillin, Q+M – Quercitin + Morin, Quercitin+Chloragenic acid)

Table 1. Mean (+SEM) MIC values of the test compounds (mg/ml) against *S. aureus* and *E. coli*

S. No	Test compound	<i>S. aureus</i>	<i>E. coli</i>
1.	Quercetin	0.658±0.12 ^a	2.18±0.20 ^{bc}
2.	Morin	1.56±0.2 ^{ab}	0.81±0.13 ^{ab}
3.	Rutin	5±0.41 ^d	2.5±0 ^{bc}
4.	Naringenin	1.25± 0 ^{ab}	1.25±0 ^{ab}
5.	Ellagic acid	2.5±0 ^{bc}	2.5±0 ^{bc}
6.	Chlorogenic acid	5±0 ^c	3.5±1.66 ^c
7.	Amoxicillin	1.14±0.1 ^{ab}	-
8.	Ciprofloxacin	-	0.0008±0.00015 ^a

Means with similar superscripts doesn't differ significantly (P>0.05)

Table 2. Agar well diffusion assay with *E. coli*

S. No.	Name of the test compound	Concentration	Area of zone of inhibition (mm ²)
1	Ciprofloxacin	10µl of 1mg/ml	1134.5
2	Ciprofloxacin+Morin	5µl of 5mg/ml +	962.5
3	Ciprofloxacin+Quercetin	5µl of 10mg/ml	1134.5
		5µl of 5mg/ml + 5µl of 10mg/ml	

Means with similar superscripts doesn't differ significantly (P>0.05)

Table 3. Agar well diffusion assay with *S. aureus*

S. No.	Name of the test compound	Concentration	Area of zone of inhibition (mm ²)
1	Amoxicillin	10µl of 5mg/ml	1213.34±68.86 ^b
2	Amoxicillin	5µl of 5mg/ml	962.50±0 ^b
3	Quercetin	10µl of 10mg/ml	86.82±11.67 ^a
4	Amoxicillin + Quercetin	5µl of 5mg/ml + 5µl of 10mg/ml	1174.91±20.17 ^b

Means with similar superscripts doesn't differ significantly (P>0.05)