IN VITRO ANTIMICROBIAL EFFICACY OF SOME PLANT EXTRACTS AGAINST MULTI-DRUG RESISTANT *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS PYOGENES* ISOLATED FROM BUFFALO MASTITIC MILK

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ABSTRACT

The conventional drugs used for the treatment of buffalo mastitis are losing their efficacy day by day due to increasing resistance in microbial organisms. It is therefore people nowadays are going back to use old but still quite potential remedy methods by using different herbs and shrubs for the treatment of different animal diseases including mastitis. In current investigation, three botanical extracts viz., garlic (*Allium sativum* L.), ginger (*Zingiber officinale*) and red chilies (*Capsicum annuum* L.) were evaluated individually and concomitantly (with ratio of 1:1) against the multidrug resistant *Staphylococcus aureus* and *Streptococcus pyogenes* isolated from buffalo mastitis. Agar well diffusion assay exhibited that red chili shown significantly (P<0.05) higher effects than garlic followed by ginger. All concentrations of red chili and 100% concentration of garlic exhibited a significantly (P<0.05) higher inhibitory effect against *Strep. pyogenes* comparing with other extracts and reference antibiotic oxacillin and streptomycin. Red chili as well as garlic in 75 and 100% concentrations also significantly (P<0.05) inhibited the *Staph. aureus* isolates comparing with ginger and reference drug. Red chili exhibited the highest inhibitory effects when combined with garlic than ginger. Garlic + red chilies showed a significantly (P<0.05) higher inhibitory effect against *Staph. aureus* and *Strep. pyogenes* as compared to other combined treatments and reference drug. Red chili as well as garlic also showed significantly (P<0.05) lower MIC (0.394 and 0.399 mg/ml respectively) against multidrug resistant *Strep. pyogenes* as compared to the ginger (0.564 mg/ml) and reference antibiotic oxacillin (0.460 mg/ml). Red chilies also showed significantly (P<0.05) lower (0.211 mg/ml) MIC against multidrug resistant *Staph. aureus* as compared to the garlic (0.391 mg/ml), ginger (0.394 mg/ml) and reference antibiotic tetracycline (0.370 mg/ml). Treatment combination based on red chili, garlic and ginger also exhibited significantly (P<0.05) lower MIC value against *Staph. aureus* and *Strep. pyogenes* as compared to ginger + garlic and reference antibiotic. This study concludes that red chili ranked 1st, garlic ranked 2nd and ginger ranked 3rd for antibacterial activity against multidrug resistant *Staph. aureus* and *Strep. pyogenes*. Treatment combination based on garlic + red chili ranked 1st, ginger + red chilies ranked 2nd and ginger + garlic ranked 3rd for antibacterial activity against multidrug resistant bacteria isolates.

Keywords: *Bubalus bubalis*, buffaloes, antimicrobial, plant extracts, multi-drug resistant,

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Staphylococcus aureus, *Streptococcus pyogenes*, buffalo mastitis

INTRODUCTION

Buffalo mastitis is a major constraint in dairy production throughout the world. It causes huge economic losses to buffalo farmers in terms of reduced production (Ebrahimi *et al*., 2007). The disease implicates as a significant constriction in dairy business and is accountable for decrements in worth and magnitude of milk and associated products (Adane *et al*., 2012). Several bacterial isolates have been identified from clinical cases of mastitis, where major etiological pathogens were Streptococcus and Staphylococcus species, while from sub-clinical cases *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae* and *Streptococcus uberis* have been recognized (Getahun *et al*., 2008). *Staphylococcus aureus* and *Streptococcus pyogenes* are known as major pathogens of mastitis that have been observed in all dairy animals (Bi *et al*., 2016).

The conventional drugs used for the treatment of mastitis are limited in types in developing countries (Dego and Tareke, 2003). The worldwide problem of antibiotic resistance has produced negative impact on antibiotic therapy thus making successful empiric therapy much more difficult to achieve. The emergence of drug resistance is an evolutionary process that is based on selection for organisms that have an enhanced ability to survive and reproduce in the presence of a drug (Suleiman *et al*., 2010). Strategies to improve the current situation include research in finding new and innovative anti-microbial sources from plants. In an attempt to reduce intensive use of antibiotics, concept of ethnoveterinary medicines is considered highly suitable to resist the bacterial activity of pathogens that develop mastitis such as *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus agalactiae*, *E. coli*, etc., (Freeman, 1997).

Herbs and spices are very important and useful as therapeutic agents against many pathological infections (Gull *et al*., 2012). These contain phytochemicals which are antimicrobial substances and are capable of attracting benefits and repel harmful organisms (Melvin *et al*., 2009). Antimicrobial agents from plant origin with selective toxicity are especially useful as a chemotherapeutic agent in treating many infectious diseases and may be of value in specific receptor attachment or it may depend on the inhibition of biochemical events essential to the pathogen, but not to the host (Omoya and Akharaiyi, 2010). In humans, about 80% and more than 90% in livestock population, plant medications are considered as the most significant and momentous and occasionally the only sources of therapeutics in various regions of the world (Mesfin *et al*., 2009). Many medicinal plants are also used to treat cows, sheep, poultry, horses and pigs (Giday *et al.,* 2009). Some medicinal plants and herbs like *Allium sativum, Bunium persicum, Oryza sativa* and *Triticum aestivum* are routinely being used by livestock farmers in rural areas to overcome the mastitis related problem (Amber *et al*., 2018; Shewit *et al*., 2012). *In vitro* antibacterial effects of herbal plants including red chili, garlic, ginger, etc., has been well documented (Obasi *et al*., 2016). However, to the best of our knowledge there is scarcity of literature on the efficacy of these plants on buffalo mastitis isolates. It is therefore, the present study was designed to evaluate the effects of some botanical extracts against multi-drug resistant (MDR) *Staphylococcus aureus* and

Streptococcus pyogenes isolated from buffalo mastitic milk samples.

MATERIALS AND METHODS

Isolation of MDR bacteria from mastitis animals

Staphylococcus aureus and *Streptococcus pyogenes* bacteria were isolated and identified from mastitis buffalo milk samples. Biochemical tests related to identification of bacterial species were performed *in vitro* as done previously (Bughti *et al*., 2017). Bacterial isolates were subjected to antibiotic sensitivity test by well diffusion method and the isolates showed resistant against more than two antibiotics of different classes were regarded as multidrug resistant and were used in current study.

Preparation of plant extracts

For comparative study of efficacy of different botanical extracts three botanicals *viz*., garlic (*Allium sativum* L.), ginger (*Zingiber officinale*) and red chilies (*Capsicum annuum* L.) were purchased from local market in fresh states. Each herb (100 g) was surface-sterilized by immersing into 70% (v/v) ethanol for 60 seconds (Kalyan, 2000). Residual ethanol on surface was evaporated in sterile laminar airflow chamber followed by homogenizing aseptically in sterile mortar and pestle. The homogenized mixture of each botanical was filtered separately through sterile cheesecloth. The extracts were considered as the 100% concentrations of the botanicals. The concentrated mother extracts were further diluted to 75% and 50% by mixing with appropriate sterile distilled water (Durairaj *et al*., 2009).

Antibacterial activity of extracts

Antibacterial activity of botanical extracts was determined by an agar well diffusion method as described by Habib *et al*. (2015). Briefly, Muller Hinton agar plates were prepared. Wells were punched with gel puncher and sealed using melted agar. Multidrug resistant (MDR) *Staph. aureus and Strep. pyogenes* culture having optical density (OD) equivalent to 0.5 McFarland units were spread over media surface. Botanical extracts of *Capsicum annuum L., Allium sativum L.* and *Zingiber officinale* were poured in the wells (50µl) in both pure form and in combined form. Plates were incubated at 37^{o}C for 24 h. Activity was determined by measuring diameter of zone of inhibitions. All tests were performed in triplicate. Zones of inhibition of botanical extracts were compared with the standards (oxacillin, streptomycin, penicillin and oxytetracycline). Water and DMSO (10%) were used as negative controls.

Minimum inhibitory concentration

The minimum inhibitory concentration was determined using micro broth dilution method (Lalitha, 2004). Serial two fold dilutions were prepared for all botanical extracts in Mueller Hinton broth (0.1ml) using a 96 well microtiter plate. Test organisms inoculated at a concentration of 2x10^{6} CFU/ml in each well of micro titer plate, whereas botanical extracts' concentrations were ranging from 0.156 mg/ml to 20 mg/ml. Well # 12 of micro plate was used as negative control having only media and well # 11 was taken as positive control by adding media and bacterial suspension. The wells' OD values were recorded by ELISA reader at 450 nm. Post 24 h incubation at 37^{o}C, the OD values again recorded. The lowest concentration showing inhibition of growth by decrease in OD value was considered the MIC of botanical extracts against test organisms.

Statistical analysis

All the collected data were entered into computer database software Microsoft Excel 2013. Furthermore, the data was compared by using the one-way ANOVA using Statistic version 8.1. The P<0.05 probability level was considered to know the significant difference between the treatments and reference antibiotics.

RESULTS

Individual inhibitory effects of botanical extracts against MDR *Streptococcus pyogenes*

The results regarding individual inhibitory effects of various botanical extracts against multidrug resistant *Streptococcus pyogenes* are presented in Table 1. The data showed that red chilies (*Capsicum annuum* L.) produced significantly (P<0.05) higher inhibitory effects against multidrug resistant *Streptococcus pyogenes* at 50% and 75% concentration as compared to garlic (*Allium sativum* L.), ginger (*Zingiber officinale*) and reference drugs oxacillin and streptomycin. While, at 100% concentration both red chilies (*Capsicum annuum* L.) and garlic (*Allium sativum* L.) significantly (P<0.05) exhibited the higher zone of inhibition as compared to ginger (*Zingiber officinale*) and reference antibiotics. Furthermore, maximum zone of inhibition against multidrug resistant *Streptococcus pyogenes* (24 mm) were measured at 100% concentration. Whereas, minimum zone of inhibition (10 mm) against multidrug resistant *Streptococcus pyogenes* was measured at 50% concentration for both ginger and garlic extracts.

Individual inhibitory effects of botanical extracts against MDR *Staphylococcus aureus*

The data presented in Table 2 showed that red chilies (*Capsicum annuum* L.) and garlic (*Allium sativum* L.) produced significantly (P<0.05) higher inhibitory effects against multidrug resistant *Staphylococcus aureus* at 50%, 75% and 100% concentration as compared to ginger (*Zingiber officinale*). The 75 and 100% concentrations of red chilies and garlic exhibited the higher inhibitory effects (P<0.05) than reference antibiotic penicillin. Red chilies also shown significantly higher inhibitory effect (P<0.05) than antibiotic streptomycin. The maximum zone of inhibition against multidrug resistant *Staphylococcus aureus* (22 mm) were measured at 100% of red chilies, whereas, minimum zone of inhibition (8 mm) was measured at 50% concentration for ginger.

Combined inhibitory effects of botanical extracts against MDR *Streptococcus pyogenes* and *Staphylococcus aureus*

Table 3 depicted the combination of 50% garlic + 50% red chilies and 50% ginger + 50% red chilies produced significantly (P<0.05) higher inhibitory effects against MDR *Streptococcus pyogenes* as compared to other combinations of botanical extracts. The combination of 50% ginger + 50% red chilies exhibited a similar zone of inhibition as shown by reference antibiotics oxacillin and streptomycin, while, 50% garlic+ 50% red chilies shown the higher (P<0.05) inhibitory effects than these reference antibiotics. Furthermore, maximum zone of inhibition (16 mm) against multidrug resistant *Staphylococcus aureus* was indicated by 50% garlic + 50% red chilies extract, which was significantly higher (P<0.05) than other combined extracts as well as reference antibiotic penicillin.

Individual MIC of botanical extracts against MDR *Staphylococcus aureus* and *Streptococcus pyogenes*

The results regarding individual minimum inhibitory concentrations (MIC) of various botanical extracts against multidrug resistant *Staphylococcus aureus* and *Streptococcus pyogenes* are presented in Figure 1. Red chilies (*Capsicum annuum* L.) showed significantly (P<0.05) lowest (0.211 mg/ml) MIC against multidrug resistant *Staphylococcus aureus* as compared to the garlic (0.391 mg/ml), ginger (0.394 mg/ml) and reference antibiotic tetracycline (0.370 mg/ml). Red chilies (0.394 mg/ml) as well as garlic (0.399 mg/ml) showed significantly (P<0.05) lowest minimum inhibitory concentrations (MIC) against multidrug resistant *Streptococcus pyogenes* as compared to the ginger (0.564 mg/ml) and reference antibiotic oxacillin (0.460 mg/ml).

Combined MIC of botanical extracts against MDR *Staphylococcus aureus* and *Streptococcus pyogenes*

The results of combined minimum inhibitory concentrations (MIC) of various botanical extracts against multidrug resistant *Staphylococcus aureus* and *Streptococcus pyogenes* are presented in Figure 2. Combination of 50% garlic +50% red chili and 50% ginger +50% red chili showed significantly lowest MIC (0.133 and 0.250 mg/ml respectively) against multidrug resistant *Staphylococcus aureus* as compared to combination of 50% ginger +50% garlic (0.321 mg/ml) and reference antibiotic tetracycline (370 mg/ml). Likewise, 50% garlic +50% red chili and 50% ginger +50% red chili showed significantly lowest minimum inhibitory concentrations (0.216 and 0.342 mg/ml respectively) against multidrug resistant *Streptococcus pyogenes* comparing with combination of 50% ginger +50% garlic (0.418 mg/ml) and reference antibiotic Oxacillin (460 mg/ml).

DISCUSSIONS

The medicinal plant and/or its parts contains biologically active substances that can be used for therapeutic purpose or can serve as a precursor for synthesis of useful drugs (Edwards, 2004). In humans about 80% and more than 90% in livestock population, plant medications are still considered as the most significant and momentous and occasionally the only sources of therapeutics in various regions (Al-Anbori *et al*., 2008). Medicinal plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties (Kalpoutzakis *et al*., 2000; Radulovic *et al*., 2010). A number of phytotherapy manuals mentioned that the various natural plants able to treat infectious diseases, fewer side effects and low toxicity (Stavri *et al*., 2009; Wiart *et al*., 2005). Antimicrobial activity of herbal extracts has been observed against anaerobic organisms as well (Souza *et al*., 2011).

In our study, highest antimicrobial potential against MDR *Staphylococcus aureus* and *Streptococcus pyogenes* was exhibited by the red chilies followed by garlic and ginger. The differences in inhibitory effects of botanical extracts in present study could be due to the characteristics and level of the antimicrobial compounds present in the extracts and their mode of action on different test microorganisms (Gull *et al*., 2012). Similar trend was also observed during combinatorial (1:1) treatments of botanical extracts. Red chili exhibited the highest inhibitory effects when combined with garlic than ginger. While lowest effect was recorded for the combination of ginger and garlic. This might be due to the facts that chilies have chemical structure of alkaloid compounds which have pungency as their chemical property that is capable of producing potent bacteriostatic and bactericidal effects on bacterial cell membrane (Lakner *et al*., 2011). The alkaloid compounds are resultant metabolic compounds produce by red chilies known as capsaicinoids. In chilies, capsaicin and dihydrocapsaicin are the major components of capsaicinoids and generally represent 90% of the chilies pungency. Capsaicinoids have been reported for their significant antimicrobial potential against ulcer causing *H. pylori* (Lakner *et al*., 2011). In another study, saponins isolated from red chilies exhibited a significant antimicrobial activity against pathogenic organisms (Iorizzi *et al*., 2002). In agreement to our results, Careaga *et al.* (2003) reported the inhibitory effects of red chili extract on *Salmonella typhimurium* and *Pseudomonas aeruginosa* inoculated to raw beef meat. In addition, both chili and capsaicin have shown marked inhibitory effects against various strains of *Vibrio cholerae* and significantly reduced the production of cholera toxin *in vitro*. The study reported that this effect is because of augmentation of the *hns* gene transcription that down regulate the transcription of *ctxAB, tcpA* and *toxT* genes (Chatterjee *et al*., 2010).

The study of Patmaraj (2000), reported the significant antimicrobial activity of ginger against *S. aureus* and *Salmonella* spp. and were on par with the findings of Indu *et al*. (2006). Gomaa and Hashish (2003) reported the inhibitory property of ginger extracts on the growth of some microorganisms including *Salmonella typhi*. Whereas, the study of Gull *et al*. (2012) reported poor susceptibility of some microbial organisms to the ginger aqueous extract. While, in a study by Melvin *et al*. (2009) the ginger extract showed moderate antimicrobial activity against *S. aureus*. These discrepancies in results probably because of susceptibility/resistant level of microbes used and/or the type of botanical (ginger) used. In a study it was observed that different varieties of onion have different antimicrobial activity, white variety has significantly higher antimicrobial activity than red variety (Adeshina *et al*., 2011). The results of antimicrobial effect of ginger in our study are in accordance with most of the reports published regarding ginger antimicrobial activity (Malu *et al*., 2008; Gull *et al*., 2012). The antimicrobial activity of ginger may be attributed to the fact that it contains antimicrobial substances such as zingiberol, zingiberine and bisabolene (Melvin *et al*., 2009). The rhizome of ginger contains pungent vanillyl ketones including gingerol and paradole, etc (Melvin *et al*., 2009). Gingerol is a mixture of crystal gingerone and it is the major cause of acidity of ginger and plays a role in inhibiting bacteria such as *S. aureus*, *Trichomonas vasmalis* and help to cure bacterial vaginosis and skin diseases (Melvin *et al*., 2009). The results obtained in our study corroborate with the report of Roy *et al*. (2006), which explains that bioactive compounds of ginger rendering antimicrobial activity are volatile in nature and antimicrobial activity of ginger extract decreases upon storage. Whereas Omoya and Akharaiyi (2011) firmed our findings by demonstrating the antimicrobial activity of the extract of ginger at the concentration of 100 mg/ml against the tested organism.

Different studies have reported that *Allium sativum* (garlic) has antiseptic and antibacterial properties because of a component named as 'allicin' (Bilal *et al*., 2009; Deeba *et al*., 2009). A recent study has also affirmed the antimicrobial potential of garlic, and also illuminated that

Table 1. Individual inhibitory effects of various botanical extracts against multidrug resistant *Streptococcus pyogenes*[1].

Concentration	**Zone of inhibition (mm)**		
	Ginger (*Zingiber officinale*)	Garlic (*Allium sativum L.*)	Red chili (*Capsicum annuum L.*)
50%	10	10	21*#
75%	12	12.4	22*#
100%	14	18*#	24*#

Significantly different in a row at P<0.05*

Significantly higher than reference antibiotic oxacillin and streptomycin at P<0.05#

[1]Zone of inhibition (mm) of reference antibiotics against *Streptococcus pyogenes* is Oxacillin (1 µg): 14; Streptomycin (1 µg): 14

Table 2. Individual inhibitory effects of various botanical extracts against multidrug resistant *Staphylococcus aureus*[1].

Concentration	**Zone of inhibition (mm)**		
	Ginger (*Zingiber officinale*)	Garlic (*Allium sativum L.*)	Red chili (*Capsicum annuum L.*)
50%	8	12*	12*
75%	10	16*^	20*#^
100%	12	16*^	22*#^

Significantly different in a row at P<0.05*

Significantly higher than reference antibiotic streptomycin at P<0.05#

Significantly higher than reference antibiotic penicillin at P<0.05^

[1]Zone of inhibition (mm) of reference antibiotics against *Staphylococcus aureus* is Streptomycin (1 µg): 16; Penicillin (1 µg): 12

Table 3. Combined inhibitory effects of various botanical extracts against multidrug resistant *Streptococcus pyogenes*[1].

Organism	Zone of inhibition (mm)				
	50% Ginger + 50% Garlic	50% Ginger + 50% Red chili	50% Garlic + 50% Red chili	Oxacillin (1 µg)	Streptomycin (1 µg)
Streptococcus pyogenes	12	14*	16**	14	14
Staphylococcus aureus	10	10	16***	12	16

*Significantly different in column at P<0.05.

**Significantly higher than reference antibiotics oxacillin and streptomycin at P<0.05.

***Significantly higher than reference antibiotic oxacillin at P<0.05.

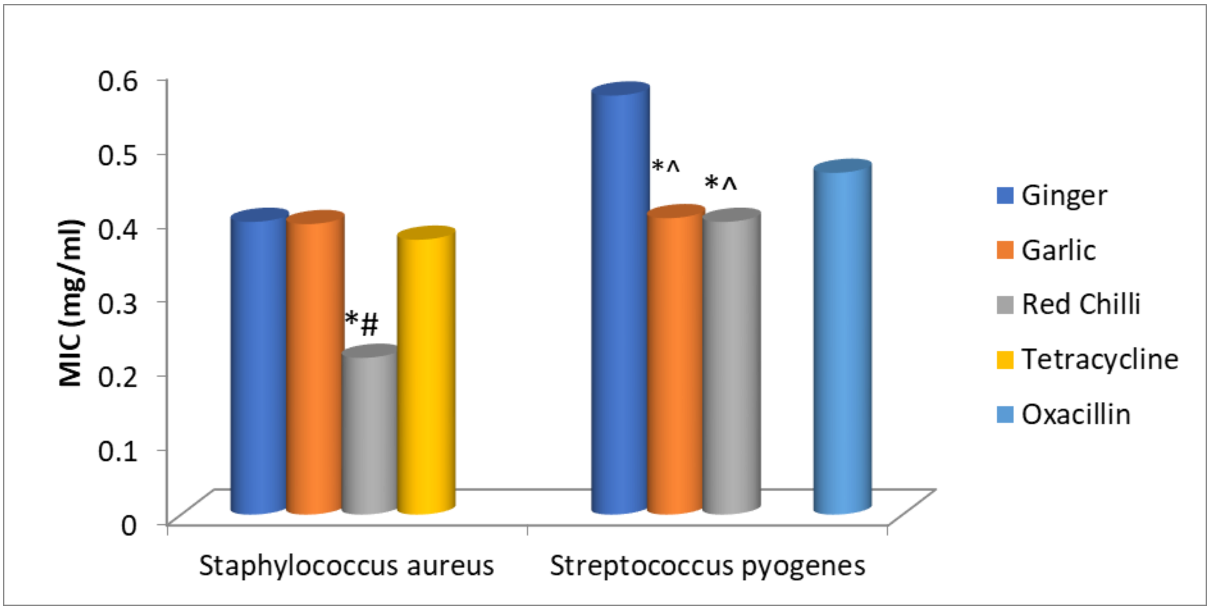

Figure 1. Individual minimum inhibitory concentrations (MIC) of various botanical extracts against multidrug resistant *Staphylococcus aureus* and *Streptococcus pyogenes*.

Significantly different from other botanical extracts at P<0.05*

Significantly different from reference antibiotic Tetracycline at P<0.05#

Significantly different from reference antibiotic Oxacillin at P<0.05^

Note: Minimum inhibitory concentration of reference antibiotics (mg/ml) against *Staphylococcus aureus*, Tetracycline: 0.370 and against *Streptococcus pyogenes*, Oxacillin: 460

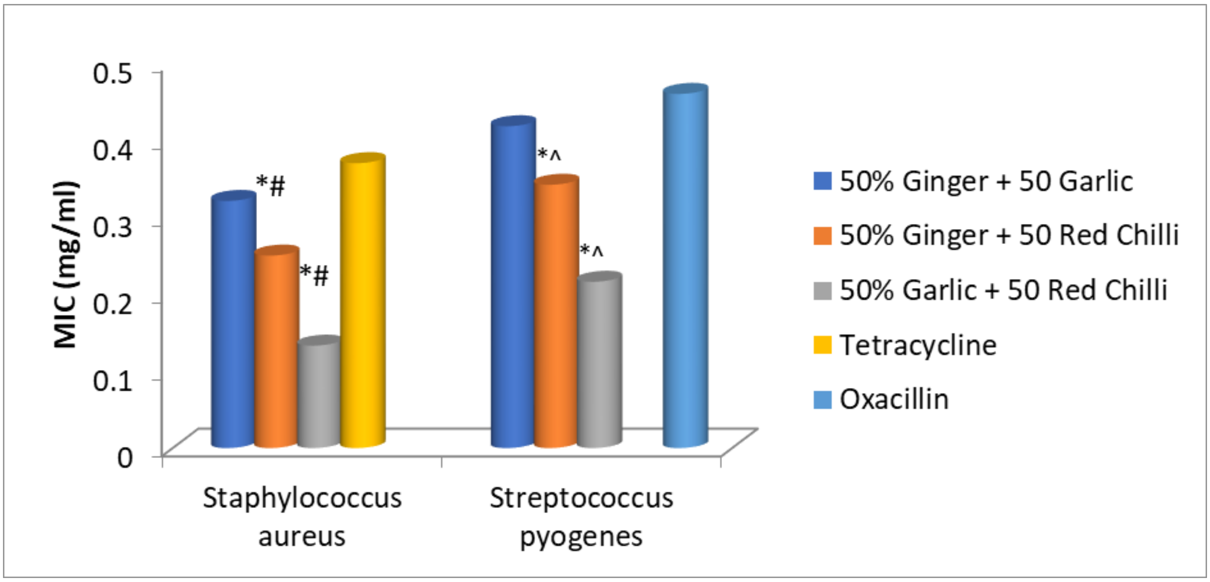

Figure 2. Combined minimum inhibitory concentrations (MIC) of various botanical extracts against multidrug resistant *Staphylococcus aureus* and *Streptococcus pyogenes.*
Significantly different from other botanical extracts at $P<0.05$*
Significantly different from reference antibiotic Tetracycline at $P<0.05$#
Significantly different from reference antibiotic Oxacillin at $P<0.05$^
Minimum inhibitory concentration of reference antibiotics (mg/ml) against *Staphylococcus aureus*, Tetracycline: 0.370 and against *Streptococcus pyogenes*, Oxacillin: 460

different varieties/genotypes have different efficacy levels depending upon the variations of their chemical constituents (Petropoulos *et al*., 2018). In a study garlic extract showed significantly higher antimicrobial activity than onion extract against the *Staphylococcus aureus*, *Salmonella enteritidis, Fusarium oxysporum, Penicillium cyclopium* and *Aspergillus niger* (Benkeblia, 2004).

It is well recognized that plant compounds (phytochemicals) may indicate extended effects when combined with each other or with other compounds (Kamboh and Zhu, 2013). This is termed as ‘effect of combination’ and in pharmacology it is known as synergism. This phenomenon has been demonstrated by several phytochemicals in terms of antimicrobial, antioxidation and immunomodulation that could be used to improve animal health and production (Kamboh *et al*., 2015; Kamboh and Zhu, 2014; Sacca-Sidi *et al*., 2016). Unfortunately, there is no literature available on the combined effects of botanicals against the growth of microbial organisms, however, several authors have reported the synergistic antimicrobial potential of botanicals when combined with synthetic antimicrobials. Betani *et al.* (2006) found the synergistic activity between the ginger ethanolic extract and antimicrobial drugs on *Staph. aureus* isolates. Likewise, in another study, synergism was observed between *P. graveolens* and antibiotic norfloxacin against many pathogenic bacteria (Rosato *et al*., 2007).

CONCLUSION

On the basis of present investigation, it was concluded that botanical extracts are highly effective even better than synthetic antibiotics against multidrug resistance buffalo mastitis organisms. Red chili ranked 1st, Garlic ranked 2nd and Ginger ranked 3rd for antibacterial activity against MDR *Staph. aureus* and *Strep. pyogenes.* Combination of 50% garlic +50% red chilies ranked 1st, 50% Ginger +50% Red Chilies ranked 2nd and 50% Ginger +50% Garlic ranked 3rd for antibacterial activity against multidrug resistance *Staph. aureus* and *Strep. pyogenes.* These results suggested that as a potential antimicrobial candidate red chili, garlic and ginger should be investigated for their *in vivo* efficacy against buffalo bacterial mastitis and/or mastitis caused by *Staph. aureus* and *Strep. pyogenes.*

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