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In vitro antibacterial activities of *Withania somnifera*, *Citrullus colocynthis* and *Piper nigrum* against subclinical mastitis bacterial pathogens of cows

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Abstract

The aim of present study was to exploring antibacterial activity of roots of *Withania somnifera* (Ashwagandha), fruit pulp of *Citrullus colocynthis* (Bitter apple fruit) and pepper corn of *Piper nigrum* (Black pepper) popularly used in the arid and semi arid area of Rajasthan, India for treatment of various ailments. Milk samples were collected aseptically from subclinical mastitis affected cows. Cultural examination of milk samples was carried out for isolation and identification of bacterial pathogens. The antibacterial activity of *Withania somnifera*, *Citrullus colocynthis* and *Piper nigrum* extracts were tested by agar cup method at different concentration viz. 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml and 15.62 mg/ml against bacterial isolates of bovine subclinical mastitis. The *Withania somnifera* and polyherb mixture showed good antibacterial activity in comparison to *Citrullus colocynthis* and *Piper nigrum* extracts against *Staphylococcus aureus*, *Staphylococcus chromogens*, *Staphylococcus epidermidis*, *Escherichia coli*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* isolated from natural cases of bovine subclinical mastitis. The alcoholic extracts *Citrullus colocynthis* and *Piper nigrum* showed moderate antibacterial activity.

Keywords: Subclinical mastitis, antibacterial activity, cows, *Withania somnifera*, polyherb

1. Introduction

Bovine subclinical mastitis (SCM) has been reported as the most important disease on dairy farms, because of the decline milk production, decreased profitability, loss due to discarded milk, treatment expenses and culling. Mastitis is caused by a wide variety of bacterial pathogens and each bacterium has differences in pathogenesis, epidemiological pattern, clinical presentation and milk losses [1]. These bacterial pathogens are generally alienated in two categories, contagious bacteria and environmental bacteria. The most common contagious pathogens are: *Staphylococcus aureus*, *Streptococcus agalactiae*, coagulase negative staphylococci, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, and *Mycoplasma* spp. Environmental bacteria survive in the surrounding environment of the cows and are considered opportunistic. The most common bacteria in this group are: *Escherichia coli*, *Citrobacter* spp., *Streptococcus uberis*, *Klebsiella* spp., *Enterobacter* spp., and *Pseudomonas aeruginosa* [2]. The animal and human diseases are treated by medicinal plants is a very old practice since prehistoric civilization. This is evident in India in Vedas between 3500 B.C and 800 B.C where medicinal properties of many plants have been elaborated. The oldest Indian literature Rig Veda mentioned several medical uses of plants and their preparations. In Ayurveda systems of medicines in India, drugs of herbal origin have been used traditionally derived either from whole plant or from leaves, stem, bark, root, flower, seed, etc. Even the Allopathic system has adopted a number of plant derived drugs which form an important segment of the modern pharmacopoeia.

The use of antimicrobial agents in animals to treat, prevent and control diseases promotes the selection and dissemination of resistant bacteria. Several study reports from different parts of the world are available on microbial resistance to antibiotics. The modern microbiological techniques demonstrate that many plants show considerable effectiveness against bacterial and fungal pathogens. Multidrug resistance is a worldwide problem accepted due to the indiscriminate use of antibiotics in animals as well as in human and failure of availability of

new drugs and vaccines. Extensive use of antibiotics on bacterial strains and lack of new antibiotics results in global problem of antimicrobial-resistance [3]. Many plant extracts showed antimicrobial activities and are used globally, but the exact principle of mechanism of action is generally obscure. Possibly lipophilic constituents disrupt the membrane of organism [4].

The present study was aimed to exploring antibacterial activity of some herbal plants popularly used in the arid and semi arid area of Rajasthan against bacterial pathogens isolated from subclinical mastitic milk of crossbred cows.

2. Materials and Methods

2.1 Collection of milk samples

After washing of udder and teats with water and dried off air the each teat was wiped off by swab of 70% alcohol. First few strips of fore milk from each teat were discarded then milk was collected from each teat in sterilized vial.

2.2 Bacterial cultural examination

The bacterial culture of each milk sample was conducted as per method suggested by Cowan and Steel [5]. The isolation and identification of bacteria was depend on morphology of colony (i.e. shape, arrangement, capsulation and presence of any other typical features), Gram's staining, motility, growth in air, spore formation and biochemical testing.

In this study, for the identification of bacteria, Hi Media ® biochemical identification kits (Hi Media Laboratories, Mumbai, Maharashtra) were used.

2.3 *In vitro* herbal extract sensitivity testing

The antibacterial activity of all the extracts was screened by agar cup method as described by Cruickshank *et al.* [6].

2.3.1 Preparations of alcoholic extracts

2.3.1.1 Collection of plant materials

In the present study, *Withania somnifera* (Ashwagandha), *Citrullus colocynthis* (Bitter apple fruit) and *Piper nigrum* (Black pepper) were used. Roots of *W. somnifera*, pulp of fruit of *Citrullus colocynthis* and *Piper nigrum* fruits (peppercorns) were purchased from the local market and identification was confirmed by botanical expert of Government Dungar College, Bikaner. They were washed with water and kept for drying in the oven at a temperature of 45-50°C for 3-5 days till the weight became constant and crushed to powder.

2.3.1.2 Soxhlet extractions

This is a hot continuous extractions procedure. The procedure was performed as described by Redfern *et al.* [7]. Five hundred gram powder of plant material was filled in porous thimbles made from filter paper with each thimble having 40-50 g of powder. These thimbles were then placed in extraction chamber. About 5 liter of 99.9% ethanol as extracting solvent was filled in bottom flask. For extraction 8 reflux cycles were performed for one batch of 500 g powder and total of 2-3 batches of 500 g powder was extracted per 5 L of solvent.

After the Soxhlet extraction, prepared crude extract was evaporated under reduced pressure by rotatory evaporator (Heidolph-instruments, Rotavapor, Germany) so that all the solvent was nearly evaporated. The concentrated extract thus obtained was left overnight at room temperature to evaporate any residual solvent. Finally, extract of a thick paste consistency was obtained which was stored in air-tight

container at 4°C in refrigerator.

2.3.2 Evaluation of *In vitro* activity of *Withania somnifera*, *Citrullus colocynthis* and *Piper nigrum* ethanolic extracts against common mastitis bacteria

The antibacterial activity of ethanolic extracts of *Withania somnifera*, *Citrullus colocynthis* and *Piper nigrum* were screened and tested against *Staphylococcus aureus*, *Staphylococcus chromogens*, *Staphylococcus epidermidis*, *Escherichia coli*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* isolated from natural cases of bovine subclinical mastitis.

2.3.2.1 Test dilution of herbal extract

The different dilutions of herbal extracts were prepared and tested. For ethanolic extract of *Withania somnifera* and *Citrullus colocynthis* dilution were prepared by dissolving prepared extract in triple glass distil water by serial dilution method to yield different concentration from 250 mg/ml to 7.812 mg/ml. For ethanolic extract of *Piper nigrum*, dilution was prepared by dissolving prepared extract in triple glass distil water with help of tween 20. Highest dilution 250 mg/ml was obtained using 20% tween 20 solution. The serial dilution was prepared using the 20% tween 20 solution in distil water to yield different concentration *viz.* 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml and 15.62 mg/ml. The diluting solution (20% tween 20) as a control was also tested for its antibacterial activity.

2.7.3 Preparation and inoculation of agar plates

Nutrient agar was used for testing *In vitro* activity of ethanolic herbal extracts. Stock inoculums of test bacterium were swept over the agar plate using a sterile cotton swab. Six equidistant wells of size 6 mm were cut into the agar. Further 100 µl of different concentrations of extract was poured into different well. One set of combination of all three herbal ethanolic extracts was prepared. They were so mixed (33.33 µl each) to achieve the same volume (100 µl) as required to show the antibacterial activity. Three replicates for each bacterial isolate were prepared. Plates were incubated at 37°C for 18-24 hrs. and zones of inhibition were measured.

3. Results and Discussion

In the present study, *In vitro* evaluation of antibacterial activity of alcoholic extracts of *Withania somnifera*, *Citrullus colocynthis* and *Piper nigrum* against isolated bacteria *Staphylococcus aureus*, *E. coli*, *Streptococcus dysgalactiae*, *Staphylococcus chromogens*, *Staphylococcus epidermis* and *Streptococcus agalactiae* from subclinical mastitis infected quarters was done. The *In vitro* antibacterial activity of ethanolic extracts was expressed in terms of zone of inhibition (Figure 1-3).

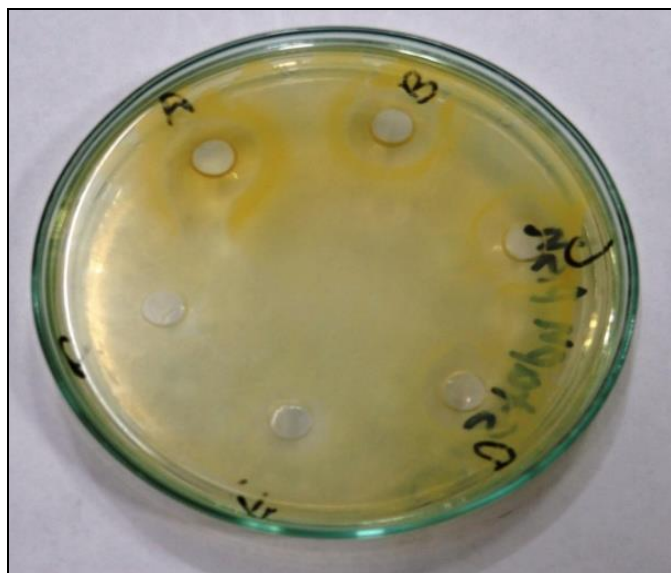
3.1 *In vitro* antibacterial activity of ethanolic extract of *Withania somnifera*

The mean±SE values of zone of inhibitions (mm) of alcoholic extract of *Withania somnifera* against subclinical mastitis isolated bacteria at different concentration are presented in Table 1. The zone of inhibition (mm) against any *Staphylococcus* spp. was not observed at the concentration of 7.812 mg/ml of ethanolic extract of *Withania somnifera*. Similarly, zone of inhibition (mm) against any *Streptococcus* spp. and *E. coli* was not observed at the concentrations of 15.625 mg/ml and 7.812 mg/ml.

Table 1: *In vitro* antibacterial activity of alcoholic extract of *Withania somnifera* against subclinical mastitis isolated pathogens at different concentrations

S. No.	Bacteria	Zone of inhibition (mm) <i>Withania somnifera</i>					
		250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml	15.625 mg/ml	7.812 mg/ml
1	<i>Staphylococcus aureus</i>	20.33±0.23	17.56±0.14	14.63±0.21	11.46±0.21	9.23±0.31	-
2	<i>Staphylococcus chromogens</i>	19.5±0.11	16.93±0.08	13.8±0.05	11.43±0.08	9.06±0.08	-
3	<i>Staphylococcus epidermidis</i>	19.23±0.03	16.36±0.08	13±0.10	11.03±0.21	5.63±2.81	-
4	<i>Streptococcus dysgalactiae</i>	17.30±0.11	15.50±0.1	12.46±0.06	8.86±0.03	-	-
5	<i>Streptococcus agalactiae</i>	17.83±0.03	15.83±0.03	12.56±0.03	8.86±0.03	-	-
6	<i>E. coli</i>	19.5±0.05	17.5±0.17	13.76±0.08	10.63±0.08	-	-

- indicates zone of inhibition not observed

**Fig 1:** Zone of inhibition of ethanolic extract of *Withania somnifera* for *Staphylococcus aureus*

Antibacterial activity of *W. somnifera* against Gram positive and Gram negative bacteria, particularly *Staphylococcus* spp., *Streptococcus* spp. and *E. coli* has been observed by many other workers in their previous *in vitro* studies [8, 9, 10, 11]. Velu and Baskaran [12] found more antimicrobial activities of ethanol extract of *Withania somnifera* against *Staphylococcus aureus*, with zone of diameter 20.10±0.17 mm. Both ethanol and aqueous extracts of *W. somnifera* are highly effective in growth inhibition of *Escherichia coli*, *Salmonella*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA) and Oxacillin resistant *S. aureus* (ORSA) [13]. Methanol and ethanol extracts of ashwagandha

leaves showed zone of inhibition 23.6 and 22.4 mm, respectively against *S. aureus* [14]. The ethanol extract showed zone of inhibition 22-24 mm against different clinical pathogens viz. *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* [15].

Iuvone *et al.* [16] suggested the possible cause of antibacterial activity that ashwagandha enhances nitric oxide synthetase activity of the macrophages, which in turn increases the microbial killing power of these immune cells resulting in enhancement of the response of cell mediated immunity (CMI). Vattem *et al.* [17] suggested that phenolics of the plants are associated with the antimicrobial effectiveness as they cause the hyperacidification of the plasma membrane interface of the pathogen which results in disruption of the H⁺ - ATPase required for ATP synthesis.

According to Kulkarni *et al.* [18] a glycoprotein glycowithanolides (WSG) commonly known as *W. somnifera* glycoprotein, isolated from the *W. somnifera* root is responsible for potent antimicrobial activity against the pathogenic bacteria and fungi. The phytochemical analysis of *W. somnifera* extracts revealed the presence of various bioactive components which might be responsible for exhibiting antibacterial activity such as alkaloids, tannins, saponins, flavinoids, glycosides, carbohydrates, terpenoids, phenolic compounds, proteins and amino acids [14].

3.2 *In vitro* antibacterial activity of alcoholic extract of *Citrullus colocynthis*

The mean ± SE values of zone of inhibitions of alcoholic extract of *Citrullus colocynthis* against subclinical mastitis isolated bacteria at different concentration are presented in Table 2. The zone of inhibition (mm) against any *Staphylococcus* spp. was not observed at the concentration of 31.25 mg/ml, 15.625 mg/ml and 7.812 mg/ml of ethanolic extract of *Citrullus colocynthis*.

Table 2: *In vitro* antibacterial activity of alcoholic extract of *Citrullus colocynthis* against subclinical mastitis isolated pathogens at different concentrations

S. No.	Bacteria	Zone of inhibition (mm) <i>Citrullus colocynthis</i>					
		250 mg/ml	125 mg/ml	62.50 mg/ml	31.25 mg/ml	15.625 mg/ml	7.812 mg/ml
1	<i>Staphylococcus aureus</i>	16.43±0.08	13.76±0.08	10.50±0.10	-	-	-
2	<i>Staphylococcus chromogens</i>	16.16±0.20	13±0.10	10.53±0.12	-	-	-
3	<i>Staphylococcus epidermidis</i>	15.43±0.08	12.73±0.03	9.53±0.06	-	-	-
4	<i>Streptococcus dysgalactiae</i>	17.33±0.03	14.43±0.08	10.46±0.03	8.83±0.03	-	-
5	<i>Streptococcus agalactiae</i>	17.5±0.05	14.56±0.03	10.5±0.10	8.43±0.08	-	-
6	<i>E. coli</i>	16.6±0.11	14.93±0.20	11.36±0.12	8.26±0.03	-	-

- indicates zone of inhibition not observed



Fig 2: Zone of inhibition of ethanolic extract of *Citrullus colocynthis* against *E. coli*

The zone of inhibition (mm) against any *Streptococcus* spp. and *E. coli* was not observed at the concentration of 15.625 mg/ml and 7.812 mg/ml of ethanolic extract of *Citrullus colocynthis*.

Similar to findings of the present study, *In vitro* antibacterial potentialities of *Citrullus colocynthis* against Gram positive and Gram negative pathogens were reported by Rodge and Biradar [19] and Nora *et al.* [20].

In vitro inhibition zone of ethanol extract of fruit of *Citrullus colocynthis* at the concentration of 500 mg/mL showed 18 mm against *Staphylococcus aureus*, 15 mm against *Bacillus cereus* [21]. Bnyan *et al.* [22] found that ethanol extract of *Citrullus colocynthis* showed maximum inhibition against *Escherichia coli* (20 mm) followed by *Proteus mirabilis* (16

mm) and *Staphylococcus aureus* (12 mm). Rezaie Keikhaie *et al.* [23] evaluated the antimicrobial activity of ethanol extract from *Citrullus colocynthis* against *S. aureus* resistant to antibiotics. The highest sensitivity was observed in concentrations of 10 and 20 mg/ml, in which was 100% bacteria were inhibited.

Tannins of *Citrullus colocynthis* prevents the growth of microorganisms by precipitating microbial protein and also makes nutritional proteins unavailable for them [24, 25]. Similarly flavonoids of *Citrullus colocynthis* are also considered as microbial inhibitor for antibiotics resistant bacteria [26] because flavonoids are known to be synthesized by plants in response to microbial infection; therefore they have potential of antimicrobial activity against a wide range of microorganisms [27].

3.3 *In vitro* antibacterial activity of ethanolic extract of *Piper nigrum*

The mean \pm SE values of zone of inhibitions (mm) of ethanolic extract of *Piper nigrum* against subclinical mastitis isolated bacteria at different concentration are presented in Table 3. The zone of inhibitions (mm) for control i.e. 20% tween 20 solution (used for making the dilutions of extract) against *Staphylococcus aureus*, *Staphylococcus chromogens*, *Staphylococcus epidermidis*, *Streptococcus dysgalactiae* (Figure 3), *Streptococcus agalactiae* and *E. coli* were 6.36 ± 0.03 , 6.33 ± 0.03 , 6.30 ± 0.01 , 6.26 ± 0.03 , 6.20 ± 0.05 and 6.36 ± 0.03 , respectively. The zone of inhibitions (mm) of ethanolic extract of *Piper nigrum* at concentrations 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml and 15.625 mg/ml were measured against all isolated bacteria. The zone of inhibitions of extract were significantly higher at all concentration for all bacteria when compare to the control values thus, indicating that the extract possess its own antibacterial property.

Table 3: *In vitro* antibacterial activity of alcoholic extract of *Piper nigrum* against subclinical mastitis isolated pathogens at different concentrations

S. No.	Bacteria	Zone of inhibition (mm) <i>Piper nigrum</i>					
		250 mg/ml	125 mg/ml	62.50 mg/ml	31.25 mg/ml	15.625 mg/ml	Control
1	<i>Staphylococcus aureus</i>	13.53 \pm 0.17 ^a	11.66 \pm 0.03 ^b	10.80 \pm 0.05 ^c	8.36 \pm 0.12 ^d	8.13 \pm 0.17 ^e	6.36 \pm 0.03 ^f
2	<i>Staphylococcus chromogens</i>	12.5 \pm 0.05 ^a	11.63 \pm 0.08 ^b	9.40 \pm 0.05 ^c	8.26 \pm 0.03 ^d	8.10 \pm 0.15 ^e	6.33 \pm 0.03 ^f
3	<i>Staphylococcus epidermidis</i>	13.46 \pm 0.08 ^a	11.60 \pm 0.05 ^b	9.46 \pm 0.06 ^c	8.16 \pm 0.03 ^d	8.03 \pm 0.08 ^e	6.30 \pm 0.01 ^f
4	<i>Streptococcus dysgalactiae</i>	11.4 \pm 0.05 ^a	10.13 \pm 0.03 ^b	8.5 \pm 0.05 ^c	8.36 \pm 0.03 ^d	7.70 \pm 0.06 ^e	6.26 \pm 0.03 ^f
5	<i>Streptococcus agalactiae</i>	11.56 \pm 0.08 ^a	10.83 \pm 0.03 ^b	8.73 \pm 0.08 ^c	8.36 \pm 0.08 ^d	7.83 \pm 0.03 ^e	6.20 \pm 0.05 ^f
6	<i>E. coli</i>	12.86 \pm 0.03 ^a	11.66 \pm 0.23 ^b	11.06 \pm 0.13 ^c	9.36 \pm 0.12 ^d	8.63 \pm 0.03 ^e	6.36 \pm 0.03 ^f



Fig 3: Zone of inhibition of ethanolic extract of *Piper nigrum* for *Streptococcus dysgalactiae*

In the present study, ethanolic extract of *Piper nigrum* showed antibacterial properties against all tested bacteria. Dorman and Deans [28] reported that *Piper nigrum* inhibited *S. aureus* as 14.5 mm. Nazia and Perween [29] published that *P. nigrum* inhibited *S. aureus* as 23 mm inhibition zone. Zone of inhibition for ethanolic extract of *Piper nigrum* for *Staphylococcus aureus* was 13 ± 0.21 mm and for *E. coli* as 12 ± 0.12 mm [30]. Similarly, Joe *et al.* [31] found ethanolic extract have antibacterial activity for *E. coli* and *Staphylococcus aureus* with the zone of inhibition ranging between 12.5 to 16 mm and 11.5 to 15.5 mm, respectively. Pundir and Jain [32] observed that ethanolic extract of black pepper showed antibacterial activity against all test bacteria with zone of inhibition ranged between 15 mm and 22 mm. According to Zarai *et al.* [33] inhibition zone of *Piper nigrum* shown 8-12 mm for *S. aureus* and 7-14 mm for *S. epidermidis*. All of extracts of *Piper nigrum* were inhibited *S.*

aureus-18, CNS-32, and CNS-36 [34]. The findings of above studies are in support of our results.

Piper nigrum contains secondary metabolites in alcoholic extract like alkaloids, flavonoids, carbohydrates, glycosides, tannins, saponins [35], terpenoids [36], phenolic compounds [37] and anthraquinones [38]. Alkaloid, tannins, phenols, terpenoid and anthraquinone have shown antibacterial properties [30, 39]. Mohammed *et al.* [40] analyzed methanolic extract of *Piper nigrum* by Gas Chromatography-Mass Spectrum techniques and found variety of organic compound. Among those compound Cyclohexene, Naphthalene, 1, 2, 3, 5, 6, 7, 8, 8a-octahydro-1,8a-dimethyl-7-(1methyl), Epiglobulol, Piperidine, 1-(1-oxo-3-phenyl-2-propenyl) and Piperine Posses antimicrobial action. Karsha and Lakshmi [41] and Zou *et al.* [42] suggested that the extracts of *Piper nigrum* changes the permeability of the cell membrane of pathogen, as a result causes metabolic dysfunction, inhibited energy synthesis and

triggered cell death.

3.4 *In vitro* antibacterial activity of alcoholic extracts in combination (poly herb)

The mean±SE values of zone of inhibitions (mm) of ethanolic extracts of combinations of *Withania somnifera*, *Citrullus colocynth* and *Piper nigrum* against subclinical mastitis isolated bacteria at different concentration are presented in Table 4. The maximum inhibition zones (mm) were observed against *Staphylococcus epidermidis* and minimum were observed against *E. coli*. The zones of inhibition (mm) for control i.e. 20% tween 20 solution (used for making the dilutions of *Piper nigrum* extract) against *Staphylococcus aureus*, *Staphylococcus chromogens*, *Staphylococcus epidermidis*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae* and *E. coli* were 6.30±0.05, 6.20±0.05, 6.23±0.06, 6.23±0.06, 6.26±0.03 and 6.20±0.05, respectively.

Table 4: *In vitro* antibacterial activity of alcoholic poly herb extract against subclinical mastitis isolated pathogens at different concentrations

S. No.	Bacteria	Zone of inhibition (mm) Polyherb					
		250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml	15.625 mg/ml	Control
1	<i>Staphylococcus aureus</i>	19.30±0.28 ^a	16.8±0.05 ^b	12.43±0.06 ^c	9.76±0.03 ^d	6.96±0.12 ^e	6.30±0.05 ^f
2	<i>Staphylococcus chromogens</i>	18.56±0.08 ^a	16.40±0.11 ^b	11.50±0.11 ^c	8.70±0.15 ^d	6.60±0.05 ^e	6.20±0.05 ^f
3	<i>Staphylococcus epidermidis</i>	19.53±0.17 ^a	15.26±1.23 ^b	11.50±0.17 ^c	8.76±0.08 ^d	6.46±0.03 ^e	6.23±0.06 ^f
4	<i>Streptococcus dysgalactiae</i>	17.46±0.08 ^a	13.50±0.11 ^b	10.20±0.05 ^c	8.23±0.03 ^d	6.53±0.06 ^e	6.23±0.06 ^f
5	<i>Streptococcus agalactiae</i>	17.33±0.08 ^a	13.93±0.13 ^b	10.33±0.03 ^c	8.33±0.08 ^d	6.53±0.03 ^e	6.26±0.03 ^f
6	<i>E. coli</i>	15.30±0.20 ^a	12.50±0.11 ^b	9.60±0.11 ^c	7.83±0.03 ^d	6.46±0.03 ^e	6.20±0.05 ^f

The zones of inhibition of combination of *Withania somnifera*, *Citrullus colocynth* and *Piper nigrum* ethanolic extracts were significantly higher at all concentration for all bacteria when compared to the values of control thus, indicating that the mix extracts of poly herbs posses a potential antibacterial property. This might be because of the secondary metabolite of all extracts in combination potentiate the antimicrobial property in comparison to alone activity.

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