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A Comparative Antibacterial Study of *Withania somnifera* Root Extract with Antibiotics against *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Ashwagandha *(Withania somnifera)* is rich in bioactive compounds and has so many beneficial properties. **Objective:** The project was designed to evaluate its current susceptibility against pathogenic organisms in comparison with the antibiotics used now-a-days. **Methodology:** Roots of the *Withania somnifera* plants were collected, washed, dried, and ground to powder. The extraction procedures were done using Chloroform in multiple phases. Antibacterial activity was done against pathogenic strains of *E. coli* and *Staphylococcus aureus* isolated from the patient's specimen by the Kirby Bauer Technique. **Results:** The average zone values against *E. coli* isolates were found to be *Withania somnifera* (28.15±2.39), followed by Ciprofloxacin (19.31±9.30), Cefotaxime (16.46±9.86), and Gentamicin (12.57±2.19). *Withania somnifera* was giving significantly higher zone than all the three drugs. Difference was significant as all the cases were with p-values <0.001. In all the cases of *E. coli* isolates, *Withania somnifera* was found to be effective and no resistance was found. While sensitivity of Ciprofloxacin, Cefotaxime, and Gentamicin, was 17.50 %, 15 %, and 2.50 % respectively. The average zone values against *Staphylococcus aureus* isolates were found to be Imipenem (31.36±5.14), followed by *Withania somnifera* (29.16±2.21), Methicillin (23.77±6.53), and Vancomycin (14.76±4.30). *Withania somnifera* and Imipenem were giving significantly higher zone than other two drugs with p-values <0.001. In all cases *Withania somnifera* was effective against *Staphylococcus aureus* isolates. Methicillin and Vancomycin showed no response in 58.33 % and 16.67 % cases respectively, showing complete resistance. Vancomycin was the most resistant and *Withania somnifera* was the most sensitive one. **Conclusion:** Results showed efficient antibacterial activity of *Withania somnifera* root extract against *Staphylococcus aureus* and *Escherichia coli*. *Withania somnifera* showed highest zone of inhibition in culture plates. Results were compared by using ANOVA and Chi- square.

Key words: *Withania somnifera*, Antibacterial activity, Bacterial Resistance, Extraction, *E. coli, Staph. aureus.*

INTRODUCTION

Bacteria cause wide variety of infections throughout the world. However widespread indiscriminate prescribing of antimicrobials has resulted in an explosion of microorganisms resistant to all common drugs[1]. The emergence of multiple drug resistance is a continuing problem. Bacteria such as *Staphylococcus aureus* and *Escherichia coli* are a continuing concern in chemotherapy because of the rising tide towards growing drug resistance[2]. *Escherichia coli* are major cause of urinary tract infections in women. These infections are complicated by the increasing prevalence of antibiotic-resistant strains of *Escherichia coli*[3].

Staphylococcus aureus continues to be one of

the commonest pathogen encountered in clinical practice, causing a number of drastic diseases[4]. Methicillin-resistant *S. aureus* (MRSA) is a major cause of hospital-acquired infections that are becoming increasingly difficult to combat because of emerging resistance to all current antibiotic classes. The hospital wards were blocked by MRSA infections. Even medical and paramedical staffs were at high risk. Most of the MRSA strains were multidrug resistant[5]. Resistance development occurs primarily among bacteria already resistant to one or more antimicrobial agents[6]. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new safe and effective therapeutic agents[7].

Withania somnifera L. Dunal (Solanaceae) commonly known as Ashwagandha is widely used in Ayurveda medicine the traditional medical system of India. This herb is used for 6000 years plus in India[8]. *Withania somnifera* is described as an herbal tonic and health food in Vedas and considered as 'Indian Ginseng' in traditional Indian system of medicine. In Ayurveda, it is classified as a rasayana (rejuvenation) and expected to promote physical and mental health, rejuvenate the body in debilitated conditions and increase longevity[9].

W. somnifera possesses therapeutic value against arthritis, stress, rheumatism, male sexual disorders and a variety of other diseases, including cancer[10]. It possesses immunomodulatory, antiserotogenic, anabolic activity, and beneficial effects in the treatment of geriatric problems[8, 11]. Plants of *W. somnifera* are rich source of bioactive compounds and secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties[12]. Toxicity studies reveal that Ashwagandha appears to be a safe compound[9].

In antibacterial studies[13] of *Withania somnifera*, old drugs were used for comparison like Chloramphenicol. We selected drugs like Ciprofloxacin, Cefotaxime, Gentamicin, Imipenem, Methicillin, and Vancomycin for comparative antibacterial studies with *Withania somnifera* root extract.

Aims and objectives

The aims and objectives of this study were to:

1. Record the antibacterial efficacy of *Withania somnifera* against *Escherichia coli* and *Staphylococcus aureus.*
2. Compare the efficacy of *Withania somnifera* with Ciprofloxacin, Cefotaxime, and Gentamicin against *Escherichia coli.*
3. Compare the efficacy of *Withania somnifera* with Imipenem, Methicillin, and Vancomycin against *Staphylococcus aureus.*

MATERIALS AND METHODS

Collection of specimen

Plants of *Withania somnifera* were collected from the grave yard Shah Payara, Rawalpindi. The plant was identified at NUST Center for Virology and Immunology Rawalpindi. Roots were selected for the extraction of Alkaloids.

Alkaloid extraction

Roots were washed, dried and ground to powder form. Equal volume of distilled water was added in powder to make the paste. Base $Ca(OH)_2$ was added till pH became basic and this mixture was left for 4-5 hours. The base converted Alkaloids into free bases. Equal volume of chloroform was added and the solution was left for whole night in shaking incubator at 37°C with the speed adjusted to 110 rpm. Next morning two phases were apparent. The aqueous phase containing acids, glycosides, sugar and pigments, was discarded. Chloroform phase containing Alkaloids, resins, fat and waxes, was collected. 10% diluted HCl was added and pH was adjusted till acidic. The selected phase was kept overnight in shaking incubator at 37°C and 110 rpm. Again two phases were obtained. Aqueous (acidic) phase containing alkaloidal salts and impurities was collected. Aqueous (acidic) phase was taken and it was made alkaline with NH_4OH till pH became basic. The solution was kept in shaking incubator for 4-5 hours at 37°C and 110 rpm and then equal volume of chloroform was added into this phase. The mixture was kept overnight in shaking incubator at 37°C at 110 rpm. The chloroform phase was separated and then evaporated using rotary evaporator and alkaloids were crystallized. Alkaloids crystals were dissolved into 5ml of chloroform to shift it to concentrator and were

concentrated into solid crystals. Alkaloids crystals were weighed in pre-weighted append-offs and aliquots were stored at -20°C for further use.

Qualitative test for alkaloids

10 mg of the extract was dissolved in 5 ml of distilled water. The solution was acidified with 2 M Hydrochloric acid. Then 1 ml of Potassium iodobismuthate solution was added. Production of orange-red precipitate was the indication of positive test[14].

Wagner's reagent test

2 g of iodine and 6 g of KI were dissolved in 100 ml of water to prepare Wagner's reagent for alkaloids. Small quantity of extract was dissolved in distilled water (ratio 0.1 g. to 1 ml.) and filtered. Then placed a maximum of one drop of alkaloid solution on a spot tile and added 5 ml warm 2 N hydrochloric acid. On adding few drops of Wagner's reagent solution, production of reddish brown color indicates the significant reaction[13].

Antimicrobial activity

Sample size

Bacterial isolates of were taken from the urine & blood samples of the patients randomly at microbiology lab, Fauji Foundation Hospital and Pakistan Institute of Medical Sciences, Islamabad. Out of sixty four, 40 Isolates were of *Escherichia coli* and 24 of *Staphylococcus aureus*. The sample size was calculated at 5% level of significance and 80% power of test. These parameters were calculated by using proportions of expected resistance for plant extract, and different drugs.

Drugs

The powdered root extract of *Withania somnifera* was dissolved in Dimethyl sulphoxide solvent which acted as Negative control. Standard Antibiotic discs of Ciprofloxacin (CIP 5), Cefotaxime (CTX 30), Gentamicin (CN 10), Methicillin (MET 5), Vancomycin (VA 30), and Imipenem (IMP 10) were used for comparison.

Determination of antibacterial activity

Nutrient agar and nutrient broth were made and Media was poured into disposable Petri plate @ 10-15 ml / plate. Nutrient broth was dispensed into sterilized glass test tubes @ 5 ml/ test tube. Broth test tubes were inoculated with bacterial isolates and placed in incubator at 37°C for 24 hours. The antibacterial activity of the root extracts of *Withania somnifera* plant was determined by the Kirby Bauer Technique. Nutrient agar plates were inoculated with the selected isolates @ 200 µl/ plate. Three standard drug discs were placed on each plate with a filter paper disc made from Gelman filter paper. The filter paper disc was impregnated with 20 µl of *Withania somnifera* root extract solution using micropipette. The plates were incubated at 37^{o}C for 18-24 hrs. The antibacterial activity was assessed by measurement of zone of inhibition in mm for the respective drug. The relative antibacterial potency of the given preparation was calculated by comparing its zone of inhibition with that of the drugs used in the studies[15].

Statistical analysis

Data was entered and analyzed by using SPSS. 16.0. Quantitative variables Zone of inhibition were compared by using **ANOVA**. Qualitatively sensitivity and resistance for each drug were reported by frequency and percentages and compared by using **Chi-square** in Gram Negative as well as Gram Positive microorganisms.

RESULTS

Different concentrations of *Withania somnifera* extract were employed against pathogenic *E. coli*. The dose optimized was 100µg/ 20µl for *Withania somnifera* extract. No clear inhibitory zones were recorded by solvent discs. The standard drug discs employed with alkaloid against *E. coli* were Ciprofloxacin, Cefotaxime, and Gentamicin (Fig. 1). The standard discs employed against *Staphylococcus aureus* were Imipenem, Methicillin, and Vancomycin (Fig. 2). All the plates were made in triplicate.

When Zone values for different drugs were compared among *E. coli* Group, the zone values appeared for 40, 18, 23 and 40 cases for *Withania somnifera*, Ciprofloxacin, Cefotaxime and Gentamicin respectively. Rest of the cases gave completely no response. The average zone values

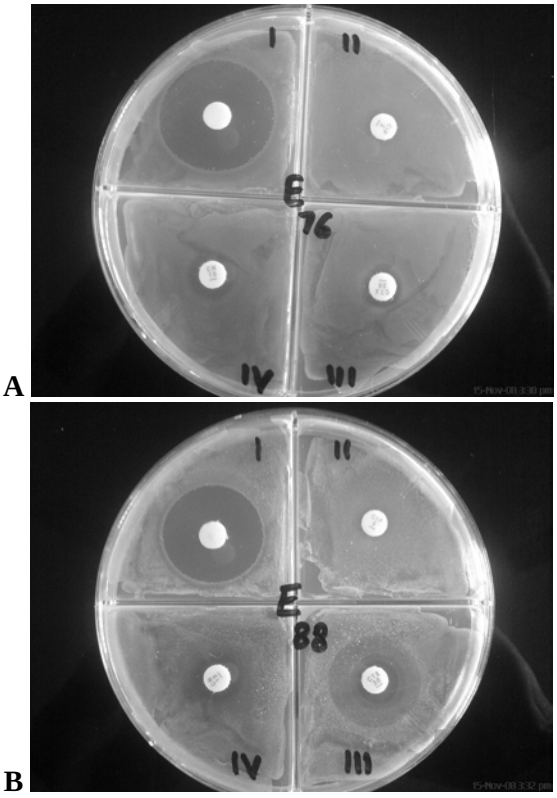

Fig. 1: Comparative Study of *Withania somnifera* extract with antibiotics against *Escherichia coli* (a) MQE 29, (b) MQE 33. I, *Withania somnifera* extract 100 µg / 20 µl; II, Ciprofloxacin 5 µg (CIP 5); III, Cefotaxime 30 µg (CTX 30); IV, Gentamicin 10 µg (CN).

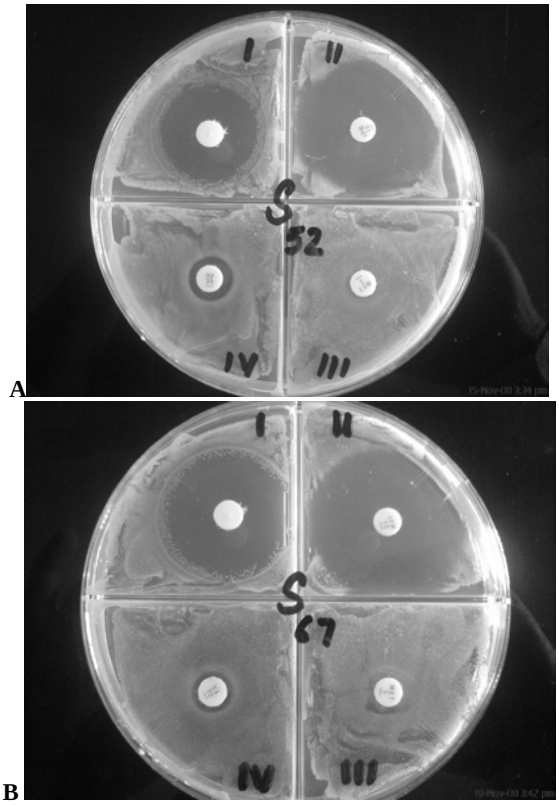

Fig. 2: Comparative Study of *Withania somnifera* extract with antibiotics against *Staphylococcus aureus* (a) MQS 99, (b) MQS 53. I, *Withania somnifera* 100 µg / 20 µl; II, Imipenem 10 µg (IMP 10); III, Methicillin 5 µg (MET 5); IV, Vancomycin 30 µg (VA 30).

were compared by using ANOVA and result found to be significant with p-value <0.001. The Highest zone value were found to be 28.15±2.39 for *Withania somnifera* and the lowest for Gentamicin *i.e.,* 12.57±2.19. While Ciprofloxacin and Cefotaxime average zone values were 19.31±9.30 and 16.46±9.86 respectively (Table 1). After Post hoc Test, *i.e.,* Tukey's test Gentamicin was significantly lower than Ciprofloxacin and Cefotaxime. *Withania somnifera* gave significantly higher zone than all the three drugs. Even no case of re-growth was found in *Withania somnifera* (Table 2).

When Zone values for different drugs were compared among *Staphylococcus aureus* Group, the zone values appeared for 24, 24, 10 and 20 cases for *Withania somnifera*, Imipenem, Methicillin and Vancomycin respectively. Rest of the cases gave completely no response. The average zone values were compared by using ANOVA and result found to be significant with p-value <0.001. The Highest zone value was found to be 31.36±5.14 for Imipenem followed by *Withania somnifera* (29.16±2.21), Methicillin (23.77±6.53), and Vancomycin (14.76±4.30) (Table 3). After Post hoc Test, *i.e.,* Tukey's test *Withania somnifera* and Imipenem were giving significantly higher zone than other two drugs. But Imipenem and *Withania somnifera* were having no significant difference (Table 4).

When comparison was made for the *E. coli* Group, it showed that the difference was significant among all drugs when a comparison made for patients who are resistant , sensitive , giving no response and having response but then re-growing., all with p-values <0.001. It was observed that in

Table 1: Comparison of zones of inhibition of drugs against *E. coli* via statistical data.

Drug	No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower bound	Upper bound		
Withania somnifera	40	28.15	2.39	0.38	27.39	28.92	24.50	32.50
Ciprofloxacin	18	19.31	9.30	2.19	14.68	23.93	8.50	33.30
Cefotaxime	23	16.46	9.86	2.06	12.20	20.72	7.80	33.20
Gentamicin	40	12.57	2.19	0.35	11.87	13.27	9.20	18.30
Total	121	19.46	8.73	0.79	17.89	21.03	7.80	33.30

Table 2: Inhibition Zone values of drugs against *E. coli* by Tukey HSD test.

Drug	No.	Subset for alpha = .05		
		1	2	3
Gentamicin	40	12.57		
Cefotaxime	23	16.46087	16.46087	
Ciprofloxacin	18		19.30556	
Withania somnifera	40			28.1525
Sig.		0.076809	0.289311	1

Means for groups in homogeneous subsets are displayed.
A. Uses Harmonic Mean Sample Size = 26.840.
B. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Table 3: Comparison of Zones of Inhibition of drugs against *Staph. aureus* via statistical data.

Drug	No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min.	Max.
					Lower bound	Upper bound		
Withania somnifera	24	29.16	2.21	0.45	28.23	30.10	22.20	32.30
Imipenem	24	31.36	5.14	1.05	29.19	33.53	15.50	35.20
Methicillin	10	23.77	6.53	2.06	19.10	28.44	12.20	33.30
Vancomycin	20	14.76	4.30	0.96	12.75	16.77	9.20	25.50
Total	78	25.45	8.01	0.91	23.65	27.26	9.20	35.20

Table 4: Inhibition Zone values of drugs against S*taphylococcus aureus* by Tukey HSD test.

Drug	No.	Subset for alpha = .05		
		1	2	3
Vancomycin	20	14.76		
Methicillin	10		23.77	
Withania somnifera	24			29.1625
Imipenem	24			31.35833
Sig.		1	1	0.472752

Means for groups in homogeneous subsets are displayed.
A. Uses Harmonic Mean Sample Size = 17.143.
B. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

resistant cases, there was none from *Withania somnifera*, 10 from Ciprofloxacin, 14 from Cefotaxime and 39 from Gentamicin. Ciprofloxacin and Cefotaxime were having similar resistance upto 25 and 30 percent. When comparison made among sensitive cases, the results were exactly opposite to

that of resistant. When Comparison made among the group of No response, we could not find any case in *Withania somnifera* as all cases were sensitive and also in Gentamicin as all cases were resistant by cutoff value of <18 for zone value, while there was a significant percentage of cases for Ciprofloxacin and Cefotaxime *i.e.,* 55 and 42.5 percent respectively, showing complete resistance (Table 5). Graph 1 is presenting the same effect.

When comparison was made for the *Staphylococcus aureus* Group, it showed that the difference was significant among all drugs, when a comparison made for patients who were resistant, sensitive , giving no response and having response but then re-growing., all with p-values <0.001. It was observed that in resistant cases there was none from *Withania somnifera,* 2 from Imipenem, 2 from Methicillin and 14 from Vancomycin. Significant percentage of Vancomycin cases were resistant by cutoff value <18 for zone value *i.e.,* 58.33 %. Imipenem and Methicillin were having similar resistance i.e., 8.33 %. When comparison made among sensitive cases the results were exactly opposite to that of resistant. 100 % (24) cases from *Withania somnifera* were sensitive. Similarly 91.67 % (22) cases from Imipenem were sensitive. 7 cases from Methicillin and 4 cases from Vancomycin were sensitive to *Staphylococcus aureus*. When Comparison made among the group of No response, we could not find any case in *Withania somnifera* as all cases were sensitive and also in Imipenem as 91.67 % cases were sensitive and 8.33 % were resistant. While there were a significant percentage of no responsive cases for Methicillin and Vancomycin i.e, 58.33 % and 16.67 % respectively, showing complete resistance (Table 6), (Fig. 3).

DISCUSSION

Plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years and there is increasing interest in plants as sources of agents to fight microbial diseases[16]. In the previous studies[10,11], various parts of the *Withania somnifera* have been reported to possess properties of medical importance. But much work was needed to evaluate its antibacterial activity.

Extraction with hexane was also done but poor results were obtained. Chloroform extracts of *Withania somnifera* root showed antibacterial activity at concentration of 20 µg/ 20 µl. So, preferred method of extraction was with chloroform. For comparison, different drugs were employed against pathogenic bacteria isolated from different patients. The reason was to check the actual current resistance status of drugs in population. Disc diffusion method was found as a convenient method for determining antibacterial activity of drugs[15]. All the conditions during antibacterial testing were standardized. Conditions such as temperature, composition of culture medium, size of inoculum, time of incubation may interfere in the results of resistance tests to drugs against pathogens.

Against all the *E. coli* isolates *Withania somnifera* were found to have good inhibitory zones significantly higher than the other drugs in the groups with P<0.001. The reason might be that this plant was not in common use for antibacterial purpose. Even no case of re-growth was found in *Withania somnifera.* Increase in resistance rates was observed to Ciprofloxacin, indicating emerging ciprofloxacin resistance among urinary tract infection isolates[17]. Statistically no significantly difference was found between Ciprofloxacin and Cefotaxime (P <0.001). 55% and 42.5% cases of Ciprofloxacin and Cefotaxime respectively, showed complete resistance with no response against *E. coli*. This resistance might be due to the most frequent use of broad-spectrum drugs Ciprofloxacin and Cefotaxime against *E. coli*[18]. Increase in resistance trends to Gentamicin were observed for the most prevalent gram-negative agents: *E. coli.* Gentamicin was frequently used in birds and animals. And its residues pass in meat and eggs to human being.

Reduced susceptibility to Vancomycin against *Staphylococcus aureus* strains collected from patient's specimen was observed[19]. Against all the *Staphylococcus aureus* isolates *Withania somnifera and* Imipenem were giving significantly higher zone than other two drugs (P-value<0.001). Imipenem was a costly antibiotic and not in common use as an antimicrobial. No case of resistance and re-growth was observed from *Withania somnifera* and all the cases were found to be sensitive against *Staph. aureus.* Vancomycin was found to be the most

Table 5: Frequency of Resistance and Sensitivity of Drugs against *Escherichia coli.*

	Withania somnifera		Ciprofloxacin		Cefotaxime		Gentamicin	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Resistant	0	0.00	10	25.00	14	35.00	39	97.50
Sensitive	40	100.00	7	17.50	6	15.00	1	2.50
No Response	0	0.00	22	55.00	17	42.50	0	0.00
Re-growth	0	0.00	1	2.50	3	7.50	0	0.00
Chi-Square	**160.00**		**31.20**		**17.33**		**149.60**	
P-Value	**0.0000**		**0.0000**		**0.0006**		**0.0000**	

Table 6: Frequency of Resistance and Sensitivity of Drugs against *Staph. aureus*

	Withania somnifera		Imipenem		Methicillin		Vancomycin	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Resistant	0	0.00	8.33	2	2	8.33	2	97.50
Sensitive	24	100.00	91.67	7	22	91.67	7	2.50
No Response	0	0.00	0.00	14	0	0.00	14	0.00
Re-growth	0	0.00	0.00	1	0	0.00	1	0.00
Chi-Square	**96.00**		**76.44**		**23.56**		**19.56**	
P-Value	**0.00**		**0.0000**		**0.0000**		**0.0002**	

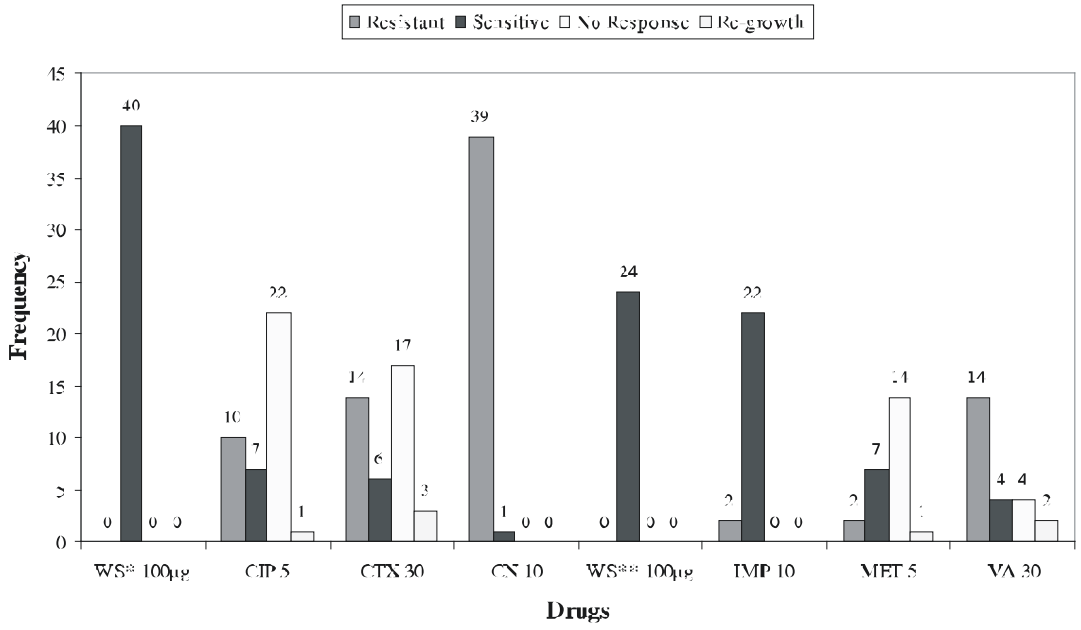

Fig. 3: Resistance status of each drug against *E. coli* and *Staph. aureus*.
*** Against *E. coli* ** Against *Staph. aureus***

resistant. Vancomycin was the preferred treatment of antibiotic-resistant gram-positive organisms in past[20]. Most isolates with reduced susceptibility to Vancomycin appeared to have developed from preexisting Methicillin-resistant *S. aureus* infections[21].

The variety of mechanisms by which bacteria acquire resistance to antimicrobial drugs is astonishing[2]. More research is urgently needed to discover more effective ways to minimize the development of resistance, to ascertain the most useful therapy for infections due to multidrug-resistant organisms[7]. There is a constant need for new and effective therapeutic agents. It is necessary to establish the scientific basis for the therapeutic actions of *Withania somnifera* as this may serve as the source for the development of more effective antimicrobial drugs.

CONCLUSION

On the basis of our results and statistical analysis it can be concluded that *Withania somnifera* extract possesses specific compounds for antibacterial properties that can be used along with other agents for the therapy of infectious diseases. The most active extracts can be subjected to isolation of the therapeutic antimicrobials and undergo further pharmacological evaluation.

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