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CHARACTERIZATION OF ROOT EXTRACTS OF *WITHANIA SOMNIFERA* AND EFFECT OF ZINC SULPHATE AND SILVER NITRATE ON ANTIBACTERIAL ACTIVITY AGAINST CERTAIN HUMAN PATHOGENIC BACTERIA

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ABSTRACT

Objectives: *There is a serious need for the expansion of new antibacterial agents from natural sources due to emergence of new infections and increase in bacterial drug resistance that have been developed over a period of time in bacteria. The present study was carried out to evaluate the effect of zinc sulphate solution on antibacterial activity of crude methanolic and aqueous extract of W. somnifera root and its phytochemical characterisation.* ***Methods:*** *Agar well diffusion assay, as well as micro broth dilution assays, were used for determination of antibacterial activity. The assay was performed in triplicate and tetracycline was used as the control. The methanolic and aqueous extracts of W. somnifera root was prepared by percolation method. Pathological isolates Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae were obtained from the Department of Microbiology, RNT Medical College & Hospital Udaipur (Raj.). Agar well diffusion method for antimicrobial susceptibility testing was performed. The antibacterial activities were assessed by the presence or absence of inhibition zones after incubating the plates at 37°C for 24 hours. Minimum inhibitory concentrations (MIC) for the selected pathogens were determined by broth micro dilution method.* ***Results:*** *Among aqueous and methanolic extracts, methanolic extract was found to possess a more potent inhibitory activity with a maximum zone of inhibition of S. aureus in antibacterial susceptibility test. MIC value of the extract for S. aureus was found to be 3.4 ±0.003 mg/ml. Among all the combinations, maximum antibacterial activity was found with methanolic extract in combination with of zinc sulphate producing a zone of inhibition of 19.00 ±0.33 mm. The antibacterial effect was similar in all combinations of aqueous extract and metal salts.* ***Conclusion:*** *Both aqueous and methanolic root extracts of W. somnifera found to possess antibacterial activity against E. coli and S.aureus. The extracts were ineffective against K. pneumoniae. The metal salts such as zinc sulphate enhances the antimicrobial potential of the root extracts. The methanol extract of W. somnifera roots possess the ability to control pathogenic bacterial growth and may be further analysed for medicinal value in ethno medicine.*

KEY WORDS

W. somnifera, root extract, $ZnSO_4$, $AgNO_3$, Antimicrobial activity

INTRODUCTION

Infectious diseases are the world's leading cause of premature deaths, killing almost 50,000 people every day. In addition to that the rate of development of resistance in pathogenic microorganisms against conventionally used antimicrobial agents is increasing with an alarming frequency [1-3]. As pathogens develop resistance to antibiotics and spread, the effectiveness of

the antibiotics gets diminished. This type of bacterial resistance to the antimicrobial agents possess a very serious threat to public health, and for all kinds of antibiotics, including the major last-resort drugs, the frequencies of resistance are increasing worldwide [4,5]. Bacterial resistance to antibiotics increases the likelihood of hospitalization, length of stay in the hospital and mortality [6].

In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world [7-11]. Most of the synthetic drugs cause side effects and also most of the microbes developed resistance against the synthetic drugs [12]. This situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and metal [13-15]. Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional systems of medicine [16]. Thus, it is a logical approach in drug discovery to screen traditional natural products. In pharmaceutical field medicinal plants are mostly used for the wide range of substances present in plants which have been used to treat chronic as well as infectious diseases [17]. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies [18]. The plant-based drugs are less toxic; side effects are scanty and also cost effective. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [19]. Apart from natural compounds from plants, metals are also identified and used as antimicrobial agents for thousand years. The use of metals as medicine was prevalent until the discovery of antibiotics [15]. Some of the metals reported with the antimicrobial activity are copper, arsenic, platinum, zinc etc. [15, 20].

Withania somnifera Dunal (Solanaceae), also known as ashwagandha or winter cherry, is one of the most valuable plants in the traditional Indian systems of medicine. This plant is used in more than 100 formulations in Ayurveda, Unani and Siddha and is believed to be therapeutically equivalent to ginseng [21]. The ethno pharmacological properties of the plant include adaptogenic, anti-sedative, and anti-convulsion activities, and the plant is used to treat various neurological disorders, geriatric, debilities, arthritis, stress and behaviour-related problems [22].*Withania somnifera* is also used as a dietary supplement because it contains a variety of nutrients and phytochemicals. A decoction of *Withania somnifera* roots and leaves is used as a nutrient and health restorative by pregnant women and the elderly. *Withania somnifera* thickens and increases the nutritive value of the milk when given to nursing mothers. Additionally, its fruits or seeds are used to curdle plant milk to make vegetarian cheeses [23].It has been reported that all of the major parts of *Withania somnifera* such as the roots, fruits and leaves provide potential benefits for human health because of their high content of polyphenols and antioxidant activities [24].The whole *Withania somnifera* plant have been reported with the antibacterial properties in methanolic extracts against different pathogenic bacteria and fungi such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans* and *Candida albicans*[25].The plant based drugs and metals have been reported with excellent antimicrobial activities, the effect of both the agents together in combination may have an enhanced effect and may prove a better therapeutic drug. Considering the vast potentiality of medicinal plants and metals as sources for antimicrobial drugs, this study was aimed to screen the aqueous and methanolic extracts of *Withania somnifera* root that could be useful for the development of new tools as antimicrobial agents for the control of infectious diseases and also investigate the effect of metal ions like zinc against these pathogenic bacteria.

MATERIALS AND METHODS

Collection of plant samples

Withania somnifera was selected among the medicinal plants for the present study to determine its antimicrobial and antioxidant potential. Healthy individual plants were located around B N University, Udaipur (Raj.) campus sites and were chosen randomly for sampling. The plant roots of *W. somnifera* were collected in sterile containers and brought to the laboratory.

Collection and maintenance of pathogenic bacteria

The pure cultures of pathogenic bacteria *Escherichia coli, Staphylococcus aureus* and *Klebsiella puemoniae* were obtained from RNT medical college Udaipur,

Rajasthan. The cultures were maintained on nutrient agar slants at an interval of one month.

Preparation of plant extracts

The collected plant root samples were washed thoroughly under running tap water until the surface adherents were removed and then shed dried at room temperature. The dried roots were powdered using mechanical grinder [26]. Two different root extracts were prepared using two different solvents viz. methanol and water. The extraction was done according to the method described by Harborne et. al [27]. Using 25g of powdered root sample, extraction was performed by soxhlet apparatus for 48hrs.The extracts were concentrated and dried by evaporation on water bath at 80°C and collected in small vials. The dry weight of extract was determined and preserved at 4-5°C in refrigerator. The percentage yield of the extract was measured by using the dry weight of extract (a) and weight of powdered root sample (b) using following formula

$$\% \text{ Yield} = \frac{a}{b} x100$$

Phytochemical profile of root extracts

The qualitative analysis of both root extracts i.e. aqueous and methanolic extracts was performed for detecting the presence of alkaloids, tannins, carbohydrates, glycosides, proteins, phenolics, flavonoids, phytosterols and terpenoids using standard procedures [27].

Determination of Total Phenolic Content

The total phenolic content of the root extracts were determined using the Folin and Ciocalteu reagent, following the method described by Singleton et al [28]. The test sample (0.2 mL) was mixed with 0.6 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent (10%). After 5 min, 1 mL of saturated sodium carbonate solution (8% w/v in water) was added to the mixture and the volume was made up to 3 mL with distilled water. The reaction mixture was incubated in the dark for 30 min and centrifuged. The supernatant was separated and was used to check the absorbance at 765 nm. The phenolic content of the extracts was calculated as gallic acid equivalents GAE/g of dry plant material on the basis of a standard curve of gallic acid (5–500 mg/L, $Y = 0.0027x - 0.0055$, $R^2 = 0.9999$). All experiments were carried out in triplicate.

Determination of Total Flavonoid Content

The flavonoid content was measured by the method described by Sathish Kumar et al [29] with minor modifications based on the formation of flavonoids-aluminium complex. The root extract (1ml, 1000µg/ml) was added to 4 mL of distilled water and was further mixed with 0.3 ml of sodium nitrite solution (5% w/v). The contents were allowed to stand for 5 min and to this mixture 0.3 ml of aluminium chloride solution (10%) was added and incubated for 1 min. After incubation, 0.2mL of 1M sodium hydroxide was added to the reaction mixture. The volume was made up to 10 mL with distilled water and the contents were mixed well. All the tubes were observed for development of yellow colour. The absorbance (O.D.) was measured at 510 nm (wavelength) in spectrophotometer (Systronics, India). Blank solution was prepared in similar way without the extract. Quercetin was used as a standard positive control of flavonoids. The assay was performed for a concentration range of 100-1000 µg/ml using the procedure described above and a standard curve of quercetin was plot. The analysis was performed in triplicate and the total flavonoid content equivalent to quercetin was calculated from standard curve. The results were expressed as mg quercetin equivalent per gram dry weight of plant extract. The mean $\pm$ standard deviation was calculated from the values obtained for total flavonoid content for each extract.

Determination of antibacterial activity of root extracts

The antimicrobial activity of *Withania somnifera* was analysed through agar well diffusion method according to Murray et al. [30]. The Muller-Hinton agar medium was poured onto the petriplates with bacterial culture (10^6 colony forming units (cfu) per mL). The wells (6mm diameter) were made by using borer. Both root extracts of *Withania somnifera* (methanolic, and aqueous) dissolved in 50% DMSO were dispensed into the wells and were allowed to diffuse for 45 min following incubation at 37°C for 24 h. The tetracycline solution (10mg/ml) and 50% DMSO were used as positive and negative control respectively. The analysis was carried out in triplicate and the sensitivity of the microbial species to the extract was determined by measuring the diameter of the microbial growth inhibition zones.

Determination of MIC

Broth micro dilution test was performed to determine MIC of the extract. 20µL of each bacterial suspension in suitable growth medium was added to the wells of sterile 96 well micro titre plate already containing 100 mg/mL of two-fold serially diluted plant extracts in proper growth medium. The final volume in each well

was 200µL. Control wells were prepared with culture medium, bacterial suspension only and plant extracts with broth only. The content of each well was mixed on a micro titre plate and were incubated for 24hrs at 37°C. After incubation 20µL of TTC (20mg/ml dissolved in distilled water) was added to each well and incubated for 30min in dark [31]. The wells were observed for appearance of red colour which is indicative of respiratory activity of the microorganism and the lowest concentration at which no colour change was observed were used for determining the MIC. The experiment was performed in triplicate.

Effect of metal salts on antibacterial activity of root extracts

The effect of metal salts on antibacterial activity of root extract was determined by agar well diffusion assay as mentioned above. The experiment was performed in triplicate. Three sets of combinations were setup, one with metals salts solution (10mg/mL) only, second with extracts only (50 mg/mL) and the third with both metal salts (10 mg/mL in case of $ZnSO_4$& 1mg/ml for $AgNO_3$) and extracts (50 mg/mL). The antimicrobial activity was determined against all three pathogenic bacteria. The plates were observed for zone of inhibition and the diameter of clear zones was noted in mm.

Results and discussion

Preparation of root extract

The extract content of methanolic root extract was found to be 8.8% with respect to the dry root powder followed by 5.2% in aqueous extract. The yield was higher in methanolic extract than aqueous.

Phytochemical screening

The preliminary phytochemical screening of plant root extracts of *W. somnifera* revealed the presence of alkaloid, tannins, carbohydrates, glycosides, phenolic compounds, flavonoids, steroids and terpenoids. This result may provide a basic idea about the phytochemical constituent of the extract. Methonolic root extract was found to contain alkaloid, carbohydrates, glycosides, phenolic compounds, flavonoids, and steroids but lack tannins, proteins and triterpenoids while aqueous extract contained alkaloid, carbohydrates, phenolic compounds and flavonoids (Table1).

Table: 1 Phytochemical profile of *W. somnifera* root extracts

S.no	Test	Methanolic extract	Aqueous extract
1	Alkaloids	+	+
2	Tannins	-	-
3	Carbohydrates	+	+
4	Glycosides	+	-
5	Proteins	-	-
6	Phenol	+	+
7	Flavonoids	+	+
8	Steroid	+	-
9	Triterpenoids	-	-

'+' Indicate presence and '–'indicate absence

Total Phenolic and Flavonoid content

The total phenolic content of *W. somnifera* in methanolic and aqueous root extract was found to be 230.00±0.001 mg GAE and 32.00 ± 0.050 mg per gram dry weight of powdered root sample respectively. Among the two extracts, methanolic extract contained the higher amount of phenolic compound than aqueous extract. The flavonoid content was also found to be higher in methanolic extract than that of aqueous extract (Table 2). Similar study with respect to phenolic and flavonoid content in 70% methanolic root extract of *W. somnifera* was done by Chaudhari et al [32]. They have reported 180.80 ± 0.01 mg/100mg extract gallic acid equivalent phenolic content and 136.97 ± 0.01 mg/100 mg extract quercetin equivalent flavonoid content they have also reported the antimicrobial and antioxidant activity is due to higher phenolic and flavonoid content. In another study by Cowan [33] shown that the antimicrobial efficacy of the herbal extract correlates with their flavonoid contents.

Antimicrobial Activity of root extract and metal salts

The antimicrobial activity of root extracts of *W. somnifera* was determined by agar well diffusion assay against *E. coli*, *S. aureus* and *K. pneomoniae*. Positive antimicrobial activity was observed in both aqueous and methanolic root extracts against *E. coli* and *S. aureus* with a zone of growth inhibition more than 9mm diameter (Table 3). No antimicrobial activity was found against *K. pneumonia*e. From the broth dilution assay it was found that, MIC of methanolic root extract was

observed 5.30 ± 0.000 and 3.40±0.03 mg/ml against *E. coli* and *S. aureus* respectively. The MIC of aqueous root extract was found to be 5.90 ± 0.000 mg/ml and 3.80± 0.05mg/ml against *E. coli* and *S. aureus* respectively (Table 4). Thus, both the extracts were found to possess similar antibacterial activity against the two-bacteria *E. coli* and *S. aureus*. The antibacterial activity of plant extracts appears to be more inhibitory to Gram-positive bacteria than Gram-negative bacteria. The antimicrobial extracts of tested plants can be assumed to be useful to the producing plant in warding off infectious diseases and there is therefore a compelling reason to suppose that anti-infective agents could be active against human pathogens as was suggested by folkloric and historical accounts [34].

Table: 2 Total Phenolic and Flavonoid content of root extracts of *W. somnifera*

S.no.	*Withania somnifera* root extracts	Total phenolic content (mgGAE/g dry weight)	Total flavonoid content (mgQE/g dry weight)
1.	Methanolic extract	230.00± 0.01	130.00± 0.01
2.	Aqueous extract	32.00± 0.05	36.00± 0.04

Values are in ± S.D mean of three replicates.

Table: 3 Antibacterial activity of root extracts of *W. somnifera* in combination with Zinc Sulphate against Human pathogens

Name of pathogenic organism	Zone of Inhibition (mm)					
	$ZnSO_4$ (10 mg/ml)	Aqueous root extract (50mg/ml)	Methanolic root extract (50mg/ml)	$ZnSO_4$ (10 mg/ml) + Aqueous root extract (50mg/ml)	$ZnSO_4$ + Methanolic root extract (50mg/ml)	Positive Control Tetracycline (1mg/ml)
E.coli	10.00 ±0.05	11.00 ±0.33	12.00±0.30	11.00 ±0.33	13.00 ±0.30	18.00 ±0.25
S.aureus	11.00 ±0.33	9.00 ±0.05	17.00±0.25	11.00 ±0.33	19.00±0.33	17.00±0.30
K. pneumoniae	10.00 ±0.33	nil	nil	10.00 ±0.33	10.00±0.33	21.00 ±0.05

Values are inhibition zone (mm) ± S.D mean of three replicates.

Table: 4 MIC of aqueous and methanolic root extracts of *W.somnifera* against Human Pathogens

Name of pathogenic organism	MIC (mg/mL)	
	Methanolic root extract	Aqueous root extract
E.coli	5.30±0.03	5.90±0.03
S.aureus	3.40±0.05	3.80±0.05

Values are in concentration (mg/mL) ± S.D mean of three replicates.

Table: 5 Antibacterial activity of root extracts of *W. somnifera* in combination with Silver Nitrate against Human pathogens

Name of pathogenic organism	Zone of Inhibition (mm)					
	$AgNO_3$ (1 mg/ml)	Aqueous root extract (50mg/ml)	Methanolic root extract (50mg/ml)	$AgNO_3$ (1 mg/ml) + Aqueous root extract 50mg/ml	$AgNO_3$ (1 mg/ml) + Methanolic root extract (50mg/ml)	Positive Control (Tetracycline) (1 mg/ml)
E.coli	10.00±0.05	11.00±0.25	12.00±0.33	11.00±0.33	11.00±0.33	18.00±0.255
S.aureus	15.00±0.33	9.00±0.33	17.00 ±0.05	10.00±0.55	14.00±0.33	17.00±0.30
K. pneumoniae	11.00±0.33	nil	nil	10.00±0.31	10.00±0.25	21.00±0.05

Values are inhibition zone (mm) ± S.D mean of three replicates.

To check the antimicrobial activity in metal salts such as $ZnSO_4$ and $AgNO_3$ a range of concentration from 1mg/ml to 50mg/ml was used to find out the minimum effective concentration. The effect of zinc and silver metal salts ($ZnSO_4$ and Ag NO_3) on microbial growth was found to be inhibitory from the antimicrobial assay. Both the salts showed positive activity against all three bacteria. A similar inhibitory effect of $ZnSO_4$ was found at 10mg/ml concentration against all three bacterial cultures (Table 3). In case of silver nitrate, the activity was found to be higher against *S. aureus* at a concentration of 10mg/ml compared to *E. coli* and *K. pneumoniae*.

In all the antibacterial assays tetracycline was used as a standard positive control. The inhibitory effects of extracts and the metal salts were compared with that of tetracycline. It was found that the antimicrobial activity in methanolic root extract at a concentration of 50mg/ml against *S. aureus* was similar to that of tetracycline at a concentration of 1mg/ml (Table 3). Apart from this, all other extracts and metal salts showed less activity than that of tetracycline against *E. coli* and *K. pneumoniae*.

Effect of combination of extract and metal salts on antimicrobial activity

The worldwide rise in bacterial infection-associated morbidity underscores the need to implement novel approaches to limit bacterial growth [35]. Antibiotics remain the treatment of choice, but their excessive and injudicious use has led to high incidences of drug and multidrug-resistant bacterial strains [36]. Moreover, agricultural use of antibiotics and bactericides results in environmental damage and can lead to widespread and unpredicted harm to wildlife [37]. It is therefore crucial to find alternatives to conventional antibiotics. In this work, we present an inexpensive and viable alternative by simply using combinations of plant root extract and metal salts. A synergistic effect of extract and metal salts was analysed against all three bacteria. The combinations of extracts with zinc sulphate have shown enhanced antibacterial activity compared to individual extract and the zinc sulphate. The antimicrobial efficacy was found to be increased on addition of $ZnSO_4$ to methanolic root extract by 8.33% against *E. coli* and 11.38% against *S. aureus*. $ZnSO_4$ when added to aqueous root extract of *W. somnifera.* It was found to enhance the antibacterial efficacy by 11.7% against *S. aureus* while it had no upshot summative effect against *E .coli* (Table 3).Comparative study of $ZnSO_4$, methanolic extract, $ZnSO_4$ and methanolic extract, aqueous root extract, $ZnSO_4$ and aqueous root extract, tetracycline revealed that $ZnSO_4$ synergistically enhanced efficacy of aqueous extract by 1% to 11% while that of methanolic extract by 8 to 11.7 % (Figure 1).

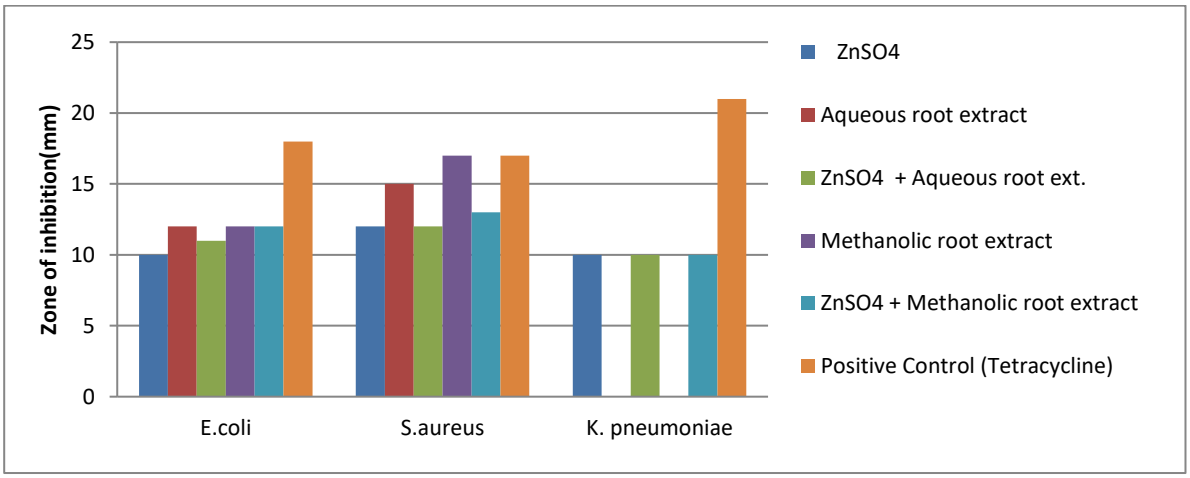

Fig : 1 Comparative antibacterial activity of *W. somnifera* root extracts with Zinc Sulphate solution

In case of silver nitrate in combination with root extract, no significant change has been observed in antimicrobial activity compared to that of root extract and silver nitrate salt individually (Figure 2). It shows that the silver salt might form complex with extracts which inhibit the antibacterial potential of *W. somnifera* root extract (Table 5).

In the present investigation, antibacterial assay of a *W. somnifera* extracts showed potent anti-bacterial activity against two out of three pathogens *viz, E. coli and S. aureus*. Maximum inhibition was observed in methanolic extract against the pathogen *Staphylococcus aureus* followed by *E. coli*. *E. coli is* an intestinal pathogenic Gram-negative bacterium causes

diarrhoea, fever, intestinal infection and urinary tract infection while S. *aureus* is the Gram-positive bacteria which causes many ailments, such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. These results suggest that *W. somnifera* is a potential source of broad-spectrum antimicrobial agents and metal salt zinc sulphate has synergistic activity when used in combination with crude extracts.

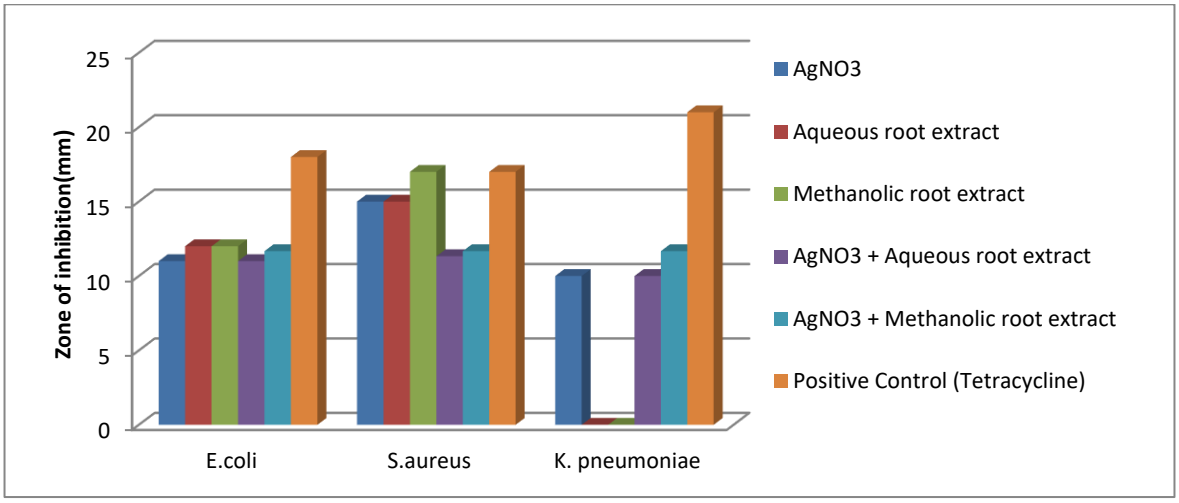

Fig : 2 Comparative antibacterial activity of *W. somnifera* root extracts with Silver nitrate solution

CONCLUSION

In this study, the antimicrobial activity of *W. somnifera* root extracts was tested against pathogenic micro-organism and the role of metal salts with these extracts. The results obtained from the study clearly suggest that the aqueous and methanolic extracts of *W. somnifera* root possess the potential antibacterial activity. Our study also reveals that the use of zinc sulphate in combination with *W. somnifera* root methanolic extract possess synergistic activity against pathogen. However, the study needs further exploration to determine the exact mechanism behind the antimicrobial activity of the chosen extract, which may enhance the pharmacological application of the selected extracts.

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