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EVALUATION OF LEAF EXTRACTS OF DIFFERENT MEDICINAL PLANTS FOR POTENTIAL ANTIBACTERIAL ACTIVITY AND PRELIMINARY PHYTOCHEMICAL ANALYSIS

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ABSTRACT

The aim of the study was to evaluate the antibacterial properties of *Datura stromanium*, *Withania somniferum, and Catharanthus roseus* by preparing their crude aqueous and organic extract against bacterial pathogens*Bacillus subtilis, Bacillus cereus,Bacillus megaterium,Eshcherichia coli, Staphylococcus aureus, Pseudomonas fluorescence, causing* diseases in human beings.The result of disc diffusion assay indicates the pattern of inhibition, depending largely upon solvent used for extraction and the organism.Organic extract provided potent antibacterial activity as compared to aqueous extract. Among all the extract methanol and ethyl acetate extract was found most active against all bacterial species. Preliminary phytochemical analysis revealed the presence of glycosides, alkaloids, phytosterols, fixed oils, phenolic compounds, carbohydrates in extracts. Further analysis shows that the active compound was not protein in nature. Antibacterial activity of crude extract of these plants was carried out to validate the use of traditional medicinal herbs.

KEY WORDS: Medicinal plants,antibacterial activity, agar disc diffusion and phytochemical analysis

INTRODUCTION

Our plant resources have a great potential to be used as antimicrobial drugs and are widely being used worldwide against number of microbial diseases.They are natural sources of antimicrobial agents primarily because of the large biodiversity of such organism and the relatively large quantity of metabolites that can extracted from them.Multi-drug resistance is a problem being faced worldwide,reason being extensive use of antibiotics, selection pressure on bacterial strains and lack of new drugs. Pathogens are increased in number (Cohen, 1992; Gold & Modellering, 1996) and develop resistance to multiple antibiotics, developing complete immunity against all antimicrobial agents and therefore be untreatable. Large number of drugs prescribed today contains plant derived active bioactive compounds. Plants have been, used for centuries as remedy for human diseases because they contain components of therapeutic value and are very diverse in molecular structure (Kaushik, 1985; Kohen& Carter, 2005). Ethanobotanical, medicinal and ornamental plants provide a rich resource for natural research and development to be used as antibiotics(Eloff,1998).In the last few years a number of studies have been conducted to verify effectiveness of plant extracts against bacterial pathogens (Perumal, 2005; Aboada *et al*., 2006; Bassam*et al*., 2006;Prashant *et al*.2006; Owen & Palombo, 2007; Pandey & Mishra 2010; Sundaram*et al*., 2011; Singh & Kumar, 2012; Jhonson *et al*., 2011; Goyal & Kaushik, 2011).

India is one of the richest countries in the world which have large diversity of medicinal plants (Kaushik , 1988).Ayurveda, a form of traditional medicine, mentions several plant species and outlines numerous medicinal uses for each.Plants are Source of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides and volatile oils(Cowan,1999).

Datura stramonium is an ayurvedic plant is antiseptic, narcotic, sedative and is useful for asthma and leaves narcotic and antispasmodic. Datura plant has been to a certain number of toxic alkaloids namely atropine, hyoscine and hycosamine, and scopolamine (Figueiredo&Esquibel 1991).

*Catharanthusroseus*is mentioned in Ayurveda and has traditionally been used to treat diseases including cancer and diabetes. The plant contain more than 70 types of alkaloids and some are known to be effective in treating various types of cancer including breast and lung cancer, uterine cancer, melanomas (EI-Sayed &


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Cordell, 1981).The anticancer drug Vincristine and vinblastine are synthesized from alkaloids of *Catharanthus roseus*.

Withania somniferum also known as Ashwagandha is traditionally known for anti-tumor, hypatoprotactive and inflammatory activities due to its abundance of secondary metabolites (Christina, 2004).*Withania somnifera* has been used as an antioxidant, adaptogen,aphrodisiac,liver tonic,anti-inflammatory agent and astringent and more recently as antibacterial,antihyperplycemic and anti-tumoral,as well as to treat ulcers and senile dementia (Rastogi and Melhotra, 1998).Most of its biological activities have been attributed the presence of group of compounds referred as withanolides (Choudhary*et al*., 1995;Sundaram*et al*., 2011)

MATERIALS AND METHODS

The test organisms*Escherichia coli, Bacillus subtilis, Bacillus cereus*, *Bacillus megaterium*are isolated from semi-arid soils of Banasthali region and were identified and characterized by morphological and biochemical identification. Bacterial strains were grown and maintained on Nutrient broth.Fresh leaves of medicinal plants *Catharanthus roseus, Datura stromanium, Withania somniferum* collected from medicinal plant garden and KrishiVigyan Kendra of Banasthali. All leaves were washed under running tap water followed by sterilized distilled water, air dried and then powdered with the help of sterilized pestle and mortar. The powders were further subjected for different extraction protocols as given below:

Aqueous extraction

5 g of air-dried powder of respective plant part viz., leaves, stem root was boiled in 200 ml distilled water till one fourth of the extract initially taken was left behind after evaporation. The solution was then filtered using muslin cloth. Filtrate was centrifuged at 5000 rpm for 15 min. The supernatant was again filtered using Whattman's filter No. 1 under strict aseptic conditions and the filtrate was collected in fresh sterilized bottles and stored at 4°C until further use.For *Catharanthus roseus* cold water extract is also prepared by this method but not boiled.

Organic solvent extraction

For Datura each 10g of leaf powder were soaked in 95% ethanol, methanol and 70% aqueous chloroform contained in three separate 500ml capacity flasks. The flasks were plugged with cotton wool, wrapped in aluminum foil, shaken vigorously and allowed to stand in the refrigerator for 24 hours. The extract obtained were evaporated to dryness using a rotatory evaporator to dryness and stored in refrigerator in reagent bottles.

For *Catharanthus roseus* 10 g of leaf powder was thoroughly mixed with 100 ml organic solvent ethanol and methanol. The mixture thus obtained was filtered through muslin cloth and then refiltered by passing through Whattman's filter No.1. The filtrate then concentrated by complete evaporation of solvent at room temperature to yield the pure extract. The appropriate amount of dried extracts dissolved with appropriate solvent at afinal concentration 100mg/ml. Each solution was stored at 4°C after collecting in sterilized glass tubes until use.

For *Withania somniferum* each 3g of leaf powder were extracted by refluxing with 25 ml ethanol, methanol, acetone, ethyl acetate, chloroform for 30 minutes and were kept overnight at room temperature before filtration. The volumes of the extracts were concentrated by evaporation until the volume of each extract became 4-5 ml (Al-Bakri*et al*., 2007)

Phytochemical screening of the extracts:

The phytochemical screening of the crude extract was carried out in order to ascertain the presence of its secondary metabolites such as saponins, alkaloids, flavonoids, streroids, tannins, glycosides, reducing sugar, carbohydrate, fixed oil, phytosterol, and phenolic compound using standard procedure (Harbone, 1973)

(A) Determination of Saponin

5ml of extract is added with 5ml of distilled water was added and shaken vigorously and warmed for 2 minutes .formation of layer of permanent foam indicates the presence of saponin. (Odebiyi & Sofawora, 1978)

(B) Determination of Tannins:

To small quantity of methanolic extract few drops of 5% $FeCl_3$ solution was added drop wise and any change in color was noted.Blue or Green color indicated the presence of tannins.

(C) Determination of Fixed oil

Spot test was done for the detection of fixed oil.In this test,small quantity of alcoholic extract was pressed

between two filter papers.Appearance of oil strain on the paper indicates the presence of fixed oil.

(D)Determination of Phenolic compound

A small quantity of the extract was dissolved in few ml of water and subjected to $FeCl_3$ test.The dilute extract was treated with dilute $FeCl_3$ solution (5%) and appearance of violet colour shows the presence of phenolic compound.

(E)Determination of Flavonoids

The extract was treated with concentrated sulphuric acid.Appearance of yellowish orange show the presence of anthocyanin's, yellow to orange show the presence of Flavones and orange to crimson show the presence of flavonones.

(F)Determination of phytosterol

Salkowski test was done for the detection of phytosterols .In this test,1ml of concentrated sulphuric acid was added to the plant extract and allowed to stand for 5 minutes.After shaking, formation of golden yellow color in the lower layer indicates the presence of phytosterols.

(G)Determination of Glycosides

Salkowski test was done for the detection of glycoside .In this test, 2ml each extract was dissolved in 2ml chloroform. Then 2ml H_2SO_4 was added carefully and shaken well .After shaking, formation of reddish brown colour indicates the presence of glycosides.

(H)Determination of Carbohydrates

A small quantity of extract was dissolved separately in 4ml of distilled water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates. The filtrate was treated with 2 to 3 drops of 1% alcoholic á-napthol solution; 2ml of concentrated Sulphuric acid was added along the sides of the tubes. Appearance of violet colored ring at the junction of two liquid shows the presence of carbohydrates.

(I)Determination of reducing sugars

Take 0.5 ml of each extract in different test tubes. Add 1ml of water and 5-8 drops of Fehling reagent was added and warm it. Appearance of brick red precipitate shows presence of reducing sugars.

(J)Determination of Alkaloids

Take 1gm of each extract in different test tubes. Add 2 drops of HCl in each test tube. Add Mayer's reagent (freshly prepared by dissolving a mixture of mercuric chloride (1.36 g) and of potassium iodide (5.00 g) in water (100.0 ml) drop by drop. A Creamy white precipitate is obtained or appearance of turbidity. Turbidity of the extract on addition of Mayer's reagent was regarded as evidence for the presence of alkaloids in the extracts (Odebiyi&Sofowora, 1978).

Antibacterial susceptibility assay

Kirby Bauer Disc diffusion assay was performed for antimicrobial screening. This was, carried out by disc diffusion technique (Bauer *et al*. 1996; Andrews, 2001). The method measures microbial growth inhibition at the surface of an inoculated medium around paper discs of Whattman's filter paper. Muller-Hinton agar was prepared as per the composition and sterilized by autoclaving at 121c 15 lbs. pressure for 15-20 minutes. The autoclaved media was poured in to sterile petriplates and allowed to solidify. Bacterial suspensions of the test microorganisms *Bacillus cereus, Bacillus subtilis,Escherichia coli*were prepared in nutrient broth and incubated at 37°C.The base plates seeded with 100 ìl inoculums of bacterial strain. Sterile filter paper discs (6mm in diameter) were prepared with Whattman's filter paper. Discs were dipped in 100 ìl of each of the extract (10mg/ml concentration) for 30 seconds, left to dry to remove residual solvent then placed on the seeded agar plates.Each extract was tested in triplicates along with positive control distilled. Thereafter plates incubated at 37°C for 24 hrs. Zone of inhibition or depressed growth of microorganisms was, measured (Plate2: Figures 7 to 12; Plate 3: Figures 13 to 18; Plate 3: Figures 19 to 24).

Extraction of proteins

This test is performed to determine that active principal responsible for antibacterial activity is protein or not. In this method protein is precipitated by adding protein denaturing agent then nonproteinated supernatant used further for antibacterial activity.Take 1ml of each plant aqueous extracts in different microfuge tubes. Then add 1 ml of Protein denaturing agent (Tannic acid 60-70%) in each microfuge, incubate for 1 hour and centrifuge at 1000rpm at 4°C for 15 minutes. Supernatant is collected .this is then applied for Lowry's to check residual proteins.Disk diffusion assay is performed for checking activity of aqueousextracts against bacterial strains. Observe the prevention of bacterial growth.

RESULTS AND DISCUSSION

In the present investigation total 14 extracts were tested, and all of them showed anti-bacterial activity. Predictions of antibacterial activity in herbal compounds extracted from leaves of plants depend largely upon the type of solvents used for extraction. Organic extract provided potent antibacterial activity as compared to aqueous extract (Thongson *et al*., 2004; Goyal *et al.,* 2012). All selected six bacterial were best inhibited by *Withania somniferum*. Most of the extracts showed good results against *Staphylococcus aureus, Pseudomonas fluorescence*, *Bacillus subtilis* and *Escherichia coli,Withania somnifera* shows maximum potential, which is greater than *Catharanthus roseus* and *Datura stramonium*. Staphylococcus aureus found to be most susceptible pathogen against all extract (Goyal *et al.,* 2012) whereas growth of Bacillus cereus and *Bacillus megaterium*not prevented by these extracts,So these strains were

TABLE: 1 COMPARITIVE STUDY OF MEDICINAL PLANTS EXTRACTS AGAINST DIFFERENT BACTERIAL STRAINS

Plant	ZONE OF INHIBITION in mm (AV±SE)						
		GRAM+VE			GRAM-VE		
	Plant extract	*B.subtilis*	*B.cereus*	*B.megaterium*	*S.aureus*	*E.coli*	*P.fluorescens*
1. Datura stramonium	Aqueous	4.25±0.47	NI	5.25±0.47	10.5±1.65	9±0.7	8±0.81
	Methanol	8.25±0.47	NI	8±1.47	24.5±1.37	24.25±1.37	10±0.64
	Ethanol	10.5±0.86	NI	3.75±0.89	25.5±1.55	12.5±0.88	8±0.81
	Chloroform	7.75±0.47	NI	NI	10.75±0.47	10.75±0.47	11.5±0.64
2. Catharanthus roseus	Hot water	5.75±0.47	NI	16.5±0.64	10±0.64	NI	3.5±0.64
	Cold water	NI	NI	NI	NI	NI	NI
	Methanol	25.25±1.88	NI	19±0.4	26.25±0.64	2.25±0.47	11.5±1.19
	Ethanol	24±1.47	NI	NI	26±1.18	NI	3.75±0.75
3. Withania somniferum	Aqueous	4.75±1.1	NI	NI	15.25±1.25	5.5±1.7	NI
	Ethanol	17.75±1.03	17±0.7	NI	11.75±1.03	11.75±1.18	4.25±0.47
	Methanol	16±9.6	7.75±1.35	NI	22±1.86	15.75±0.8	3.5±1.04
	Chloroform	22±0.8	15±1	NI	19.5±1.55	13±0.65	2.25±0.47
	Acetone	16.75±2.32	12.75±0.75	NI	21.75±0.85	12.25±0.62	8.25±0.62
	Ethyl acetate	22.75±1.31	15±1	NI	26.5±4.19	18.25±1.54	5±0.64
4. Negative control	Distilled water	NI	NI	NI	NI	NI	NI

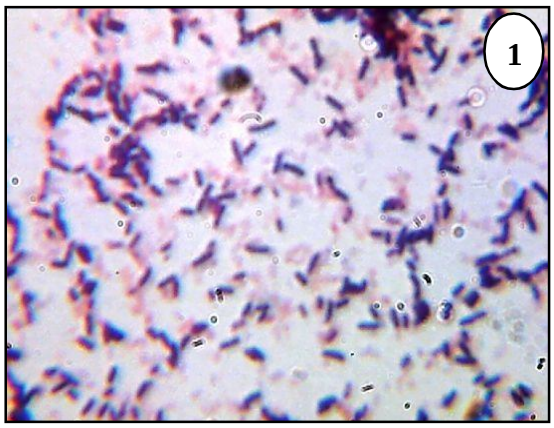
TABLE: 2 ANTIBACTERIAL ACTIVITIES OF AQUEOUS EXTRACTS OF MEDICINAL PLANTS AFTER DENATURATION OF PROTEINS

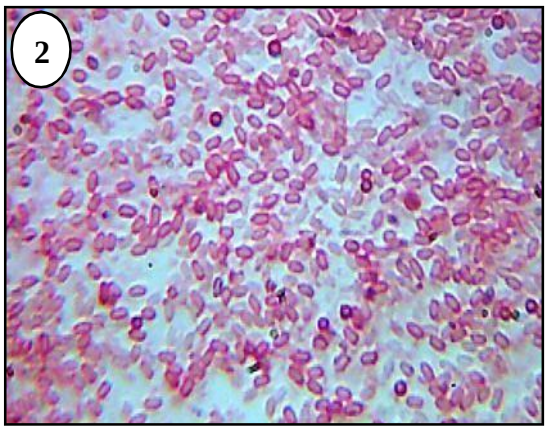
PLANT	**Sample**	**PROTEIN (µg/ml)**	**ZONE OF INHIBITION (AV±SE)**					
			B.subtilis	*B.cereus*	*B.megaterium*	*S.aureus*	*E.coli*	*P.fluroscence*
1. Datura stramonium	Crude	446	4.25±0.47	NI	5.25±0.47	10.5±1.65	9±0.7	8±0.81
	Denatured	60.34	13.5±0.95	17±0.4	16±0.4	8±1.08	12.5±0.5	14.75±0.75
2. Catharanthus Roseus	Crude	534.23	5.75±0.47	NI	16.5±0.64	10±0.64	NI	3.5±0.64
	Denatured	53.8	13.5±0.64	18±5.75	15±0.7	6.5±1.55	11.5±0.64	15.5±0.64
3.Withania somniferum	Crude	180.76	4.75±1.1	NI	NI	15.25±1.25	5.5±1.7	NI
	Denatured	45.39	12±0.7	21.25±0.62	12.50±0.8	8±0.4	12.75±0.75	17.5±0.64

TABLE: 3 PHYTOCHEMICAL COMPOUNDS OF LEAVES OF PLANTS

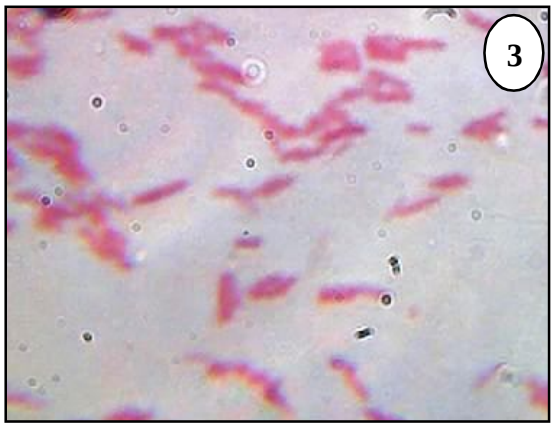
S.NO.	**PHYTOCHEMICAL TEST**	***Withania somniferum***	***Catharanthus roseus***	***Datura stromanium***
1.	**PHENOLIC TEST**	+	++	-
2.	**GLYCOSIDES TEST**	++	+	±
3.	**SAPONIN TEST**	+	+	+
4.	**ALKALOIDS TEST**	++	++	++
5.	**REDUCING SUGAR TEST**	±	±	±
6.	**FLAVONOID TEST**	+	+	-
7.	**PHYTOSTEROL TEST**	+	+	+
8.	**CARBOHYDRATE TEST**	+	+	±
9.	**FIXED OIL TEST**	+	+	+
10.	**TANNINS TEST**	-	+	+

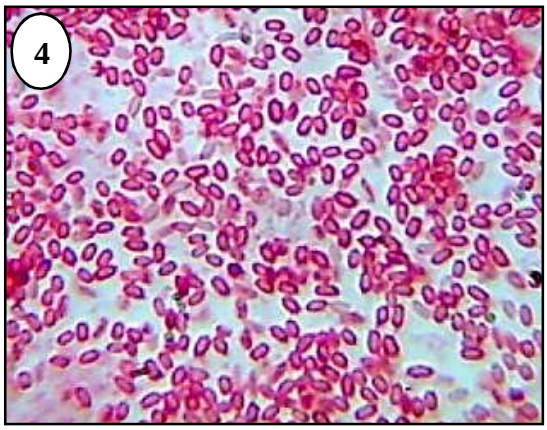
(+) Positive: (++) strongly positive; (±) Trace; (-) Not detected

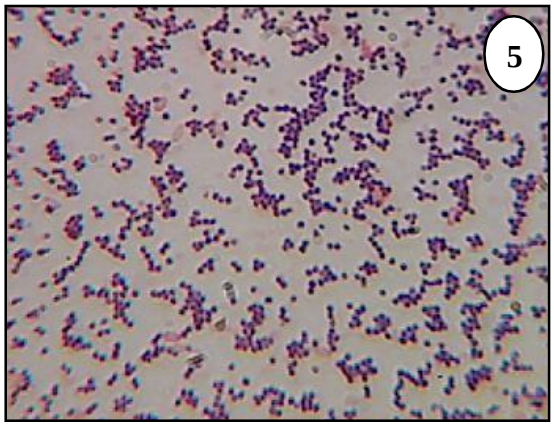


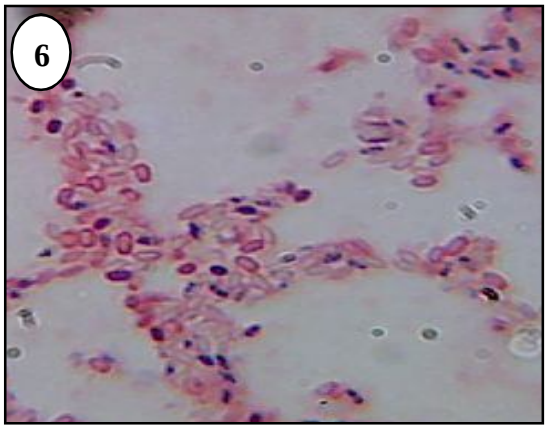


EXPLANATION OF FIGURES 1 to 6 :
Plate showing Gram staining of the isolated Bacterial species
(1) *B.Cereus*
(2) B.subtilis
(3) Bacillus megaterium
(4) E. coli
(5) S.aureus
(6) P. fluorescens

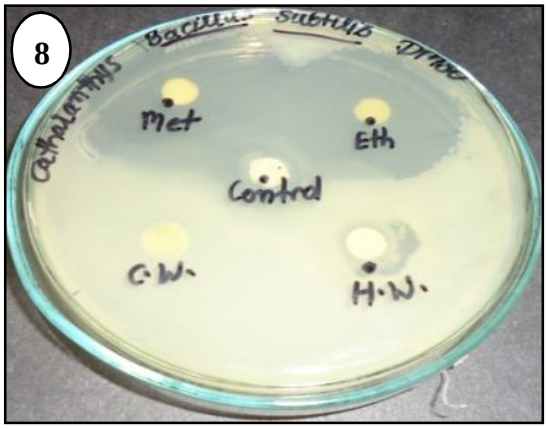


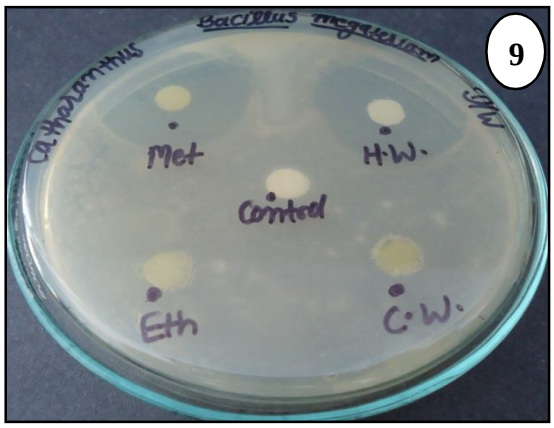


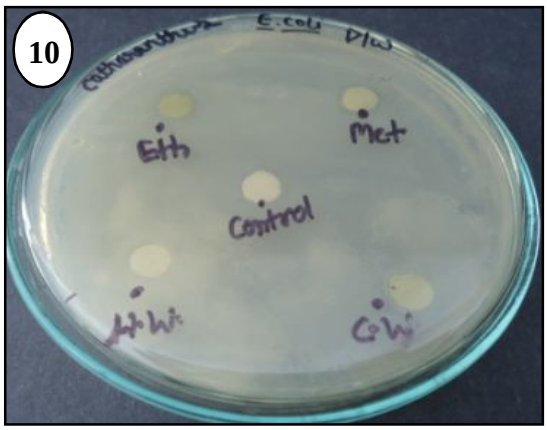




EXPLANATION OF FIGURES 7 to 8 :
Plates showing antibacterial activity of *Catharanthus roseus*
(7) .B. cereus,
(8).B. subtilis,







EXPLANATION OF FIGURES 9 to 12 :

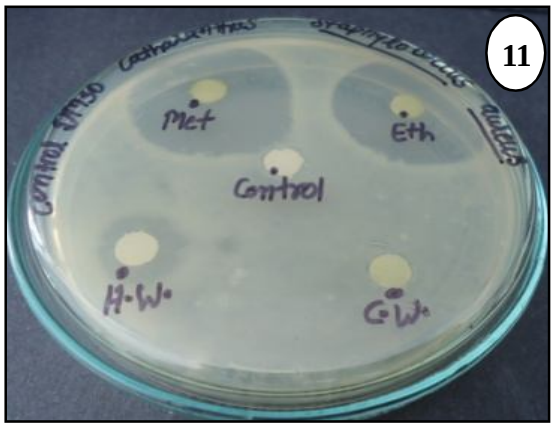
Plates showing antibacterial activity of *Catharanthus roseus*

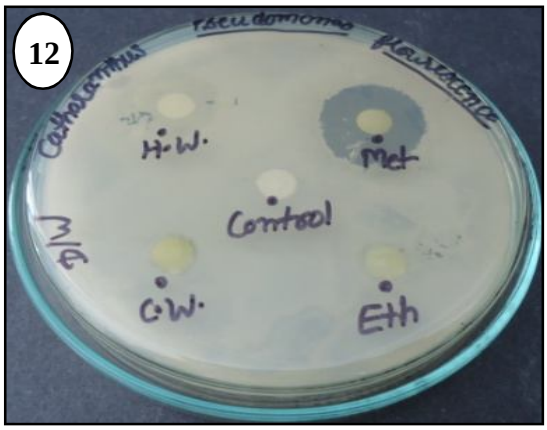
(9) B. megaterium

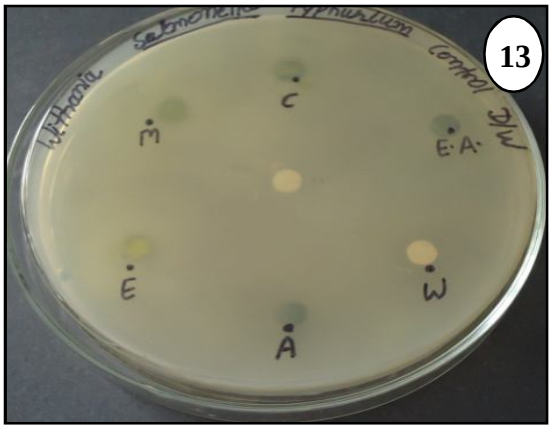
(10) E. coli

(11) S. aureus,

(12) P. fluorescens







EXPLANATIONS OF FIGURES 13 to 16 :

Plates showing antibacterial activity of *Withania somniferum*

(13) B. cereus,

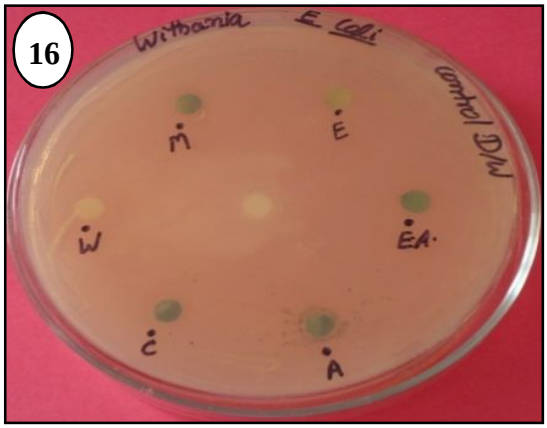
(14) B. subtilis,

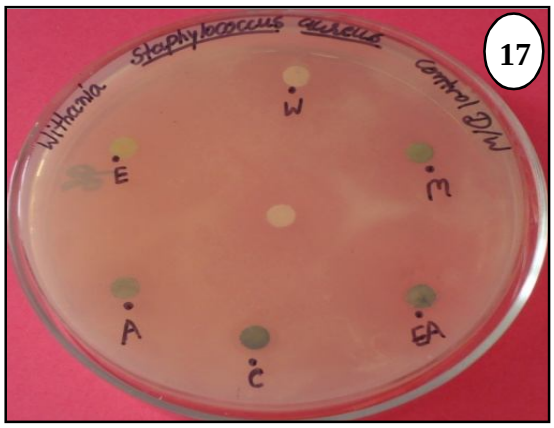
(15) B. megaterium

(16) E. coli

(17) S. aureus,

(18) P. fluorescens



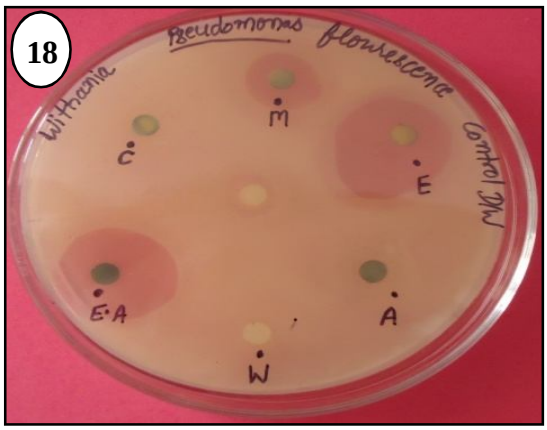


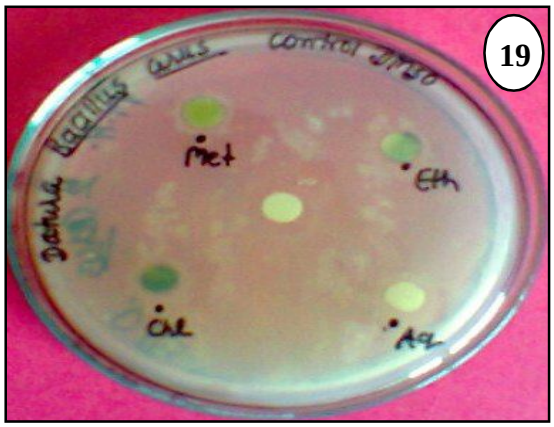
EXPLANATIONS OF FIGURES 17 to 18 :

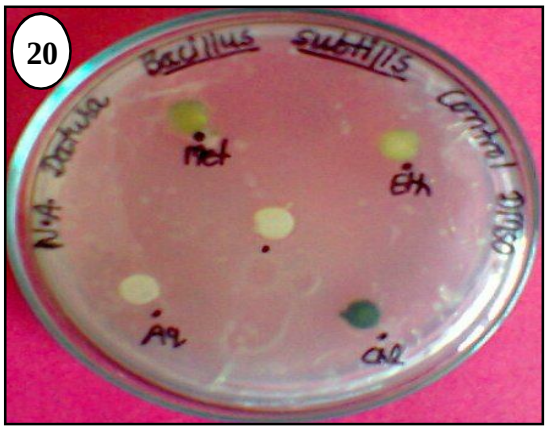
Plates showing antibacterial activity of *Withania somniferum*

(17) S. aureus,

(18) P. fluorescens







EXPLANATIONS OF FIGURES 19 to 24 :

Plates showing antibacterial activity of *Datura stramonium*

(19) B. cereus,

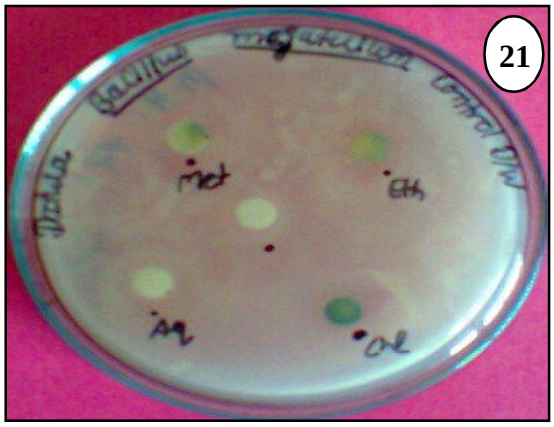
(20) B. subtilis,

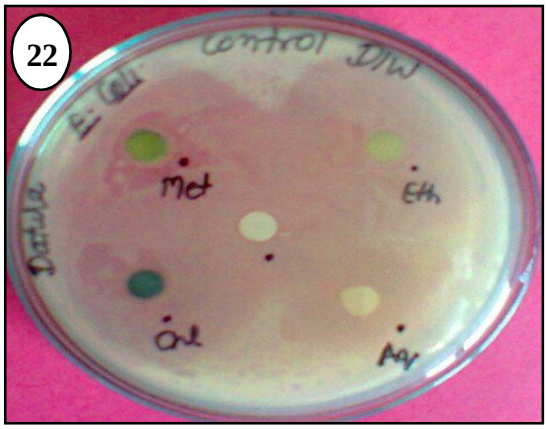
(21) B. megaterium

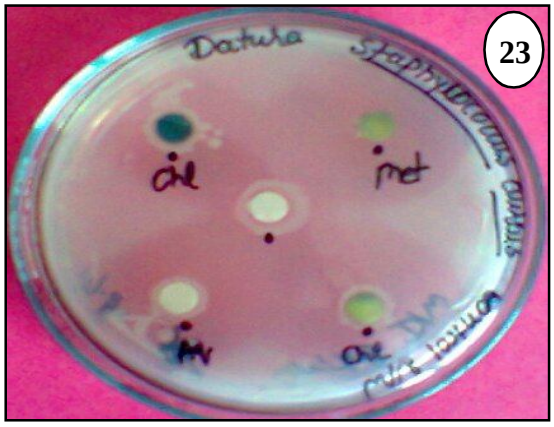
(22) E. coli

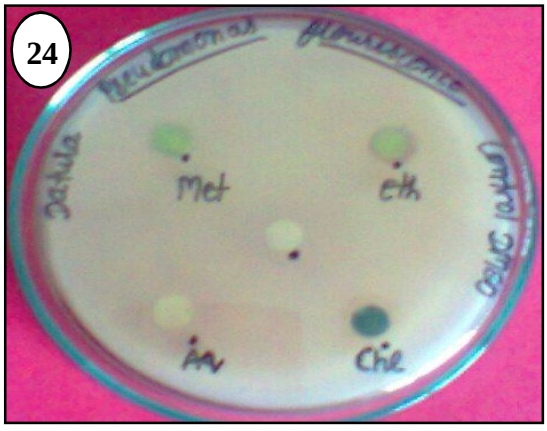
(23) S. aureus,

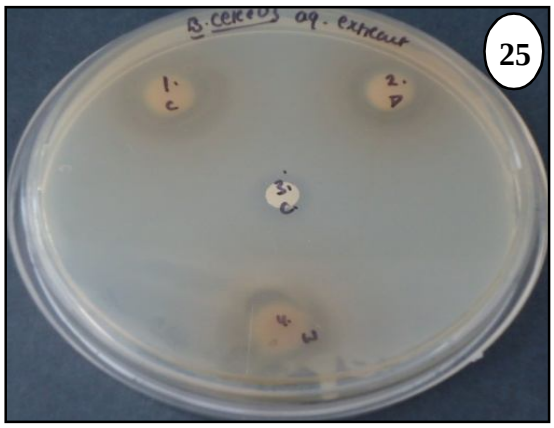
(24) P. fluorescens

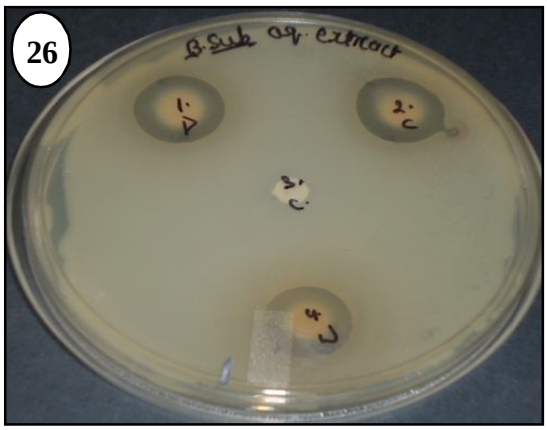


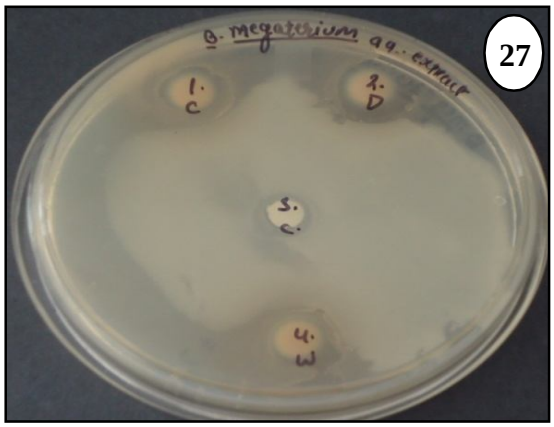












EXPLANATIONS OF FIGURES 25 to 30 :

Plates showing antibacterial activity of aqueous extracts after denaturation of proteins

(25) B. cereus,

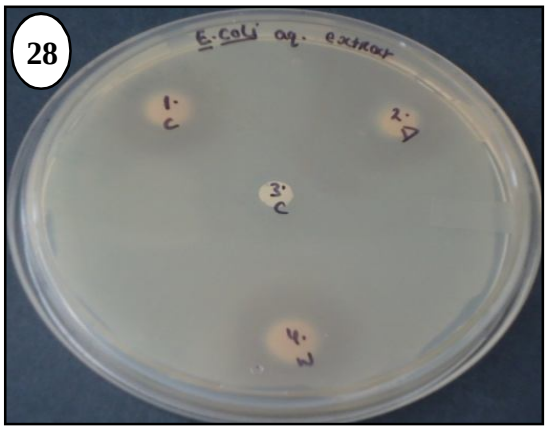
(26) B. subtilis,

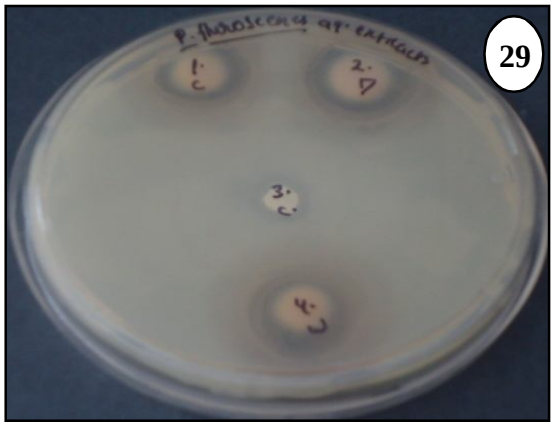
(27) B. megaterium

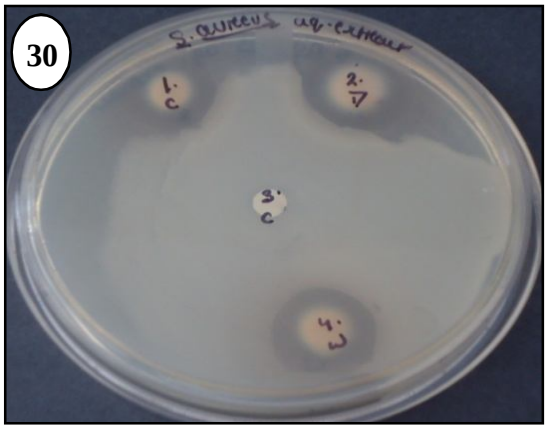
(28) E. coli

(29) S. aureus,

(30) P. fluorescens







observed as less susceptible. The maximum inhibition zone (26.5±4.19) was, observed by Ethyl acetate Extract of *Withania somniferum* against *Staphylococcus aureus*, for *Datura stramonium* ethanol extract shows maximum zone of inhibition against *Staphylococcus aureus* (25.5±1.55). Methanol extract of *Catharanthus roseus* shows maximum inhibition zone (26.25±0.64) against *Staphylococcus aureus* .Whereas the ethyl extract of *Withania somniferum* shows good result against maximum strain of pathogenic bacteria selected for the study. Furthermore, Gram-positive bacteria showed good results than gram-negative bacteria. This is probably due to the differences in chemical composition and structures of Cell walls of both types of microganisms (Goyal *et al.,* 2012). The data pertaining to the antibacterial potential tabulated in (Figure: 31,32 &33; Table: 1).

We observed that the aqueous extract is not susceptible for bacterial strains. After removal of protein aqueous extract showed activity against bacterial pathogens. Thisindicates that active principal is not protein and is not responsible for antibacterial potential.

According to Table:2 aqueous extract of *Withania somniferum* showed highest activity against all microbial strains.Bacillus cereus is most susceptible bacteria which is showing maximum zone of inhibition (21.25 ± 0.62 mm).*S. aureus* shows minimum zone of inhibition for *Catharanthus roseus*extract (6.5 ± 1.55 mm).These

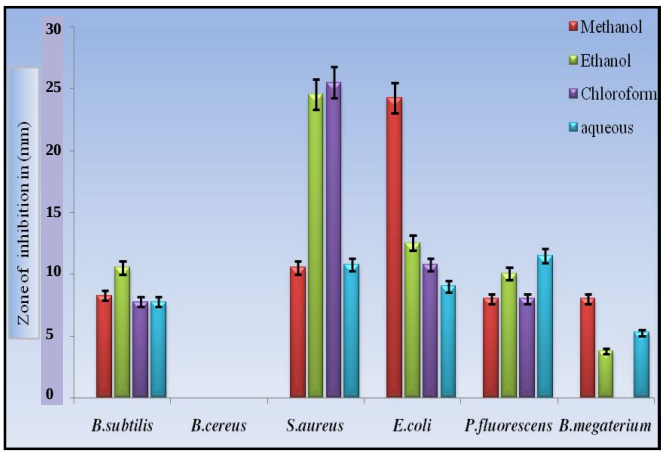


Figure 31: Antibacterial activity of *Datura stramonium*

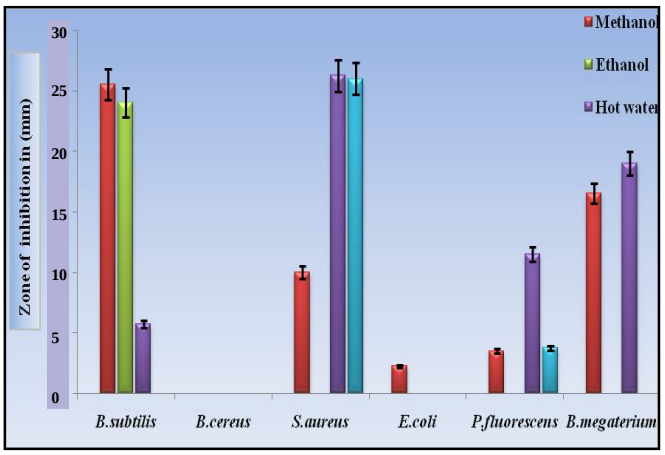


Figure 32: Antibacterial activity of *Catharanthus roseus*

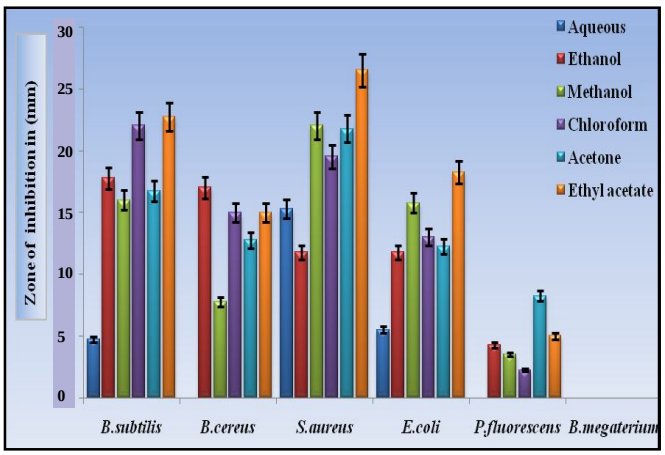


Figure 33: Antibacterial activity of *Withinia somniferum.*

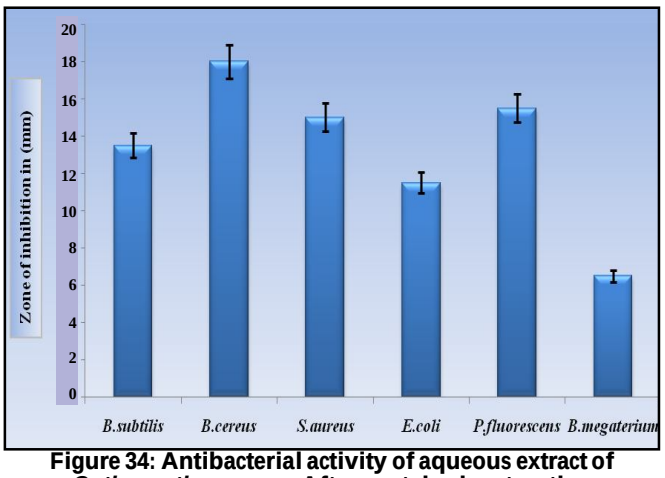


Figure 34: Antibacterial activity of aqueous extract of *Catharanthus roseus* After protein denaturation

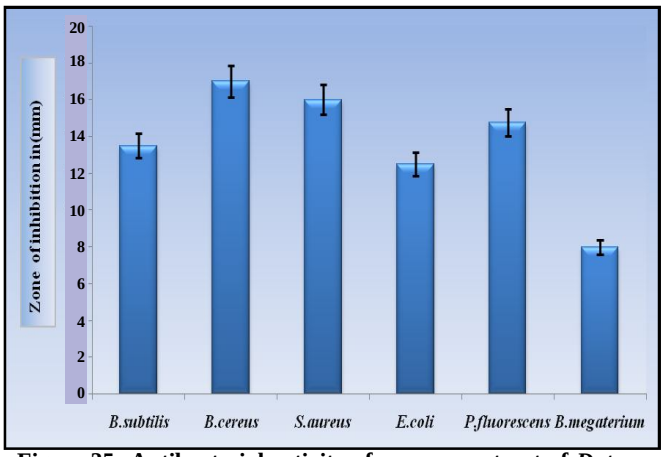


Figure 35: Antibacterial activity of aqueous extract of *Datura stramonium*,after protein denaturation

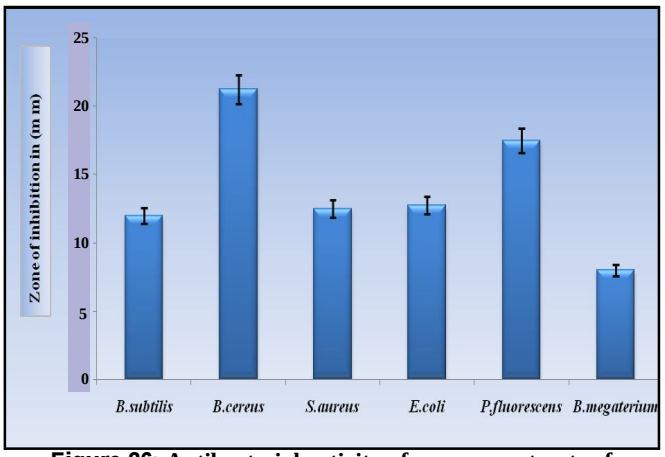


Figure 36: Antibacterial activity of aqueous extracts of *Withania somniferum* after protein denaturation

results indicates that there are some other compounds which are responsible for antibacterial activity.For revolving this reason photochemical analysis will be prevailed for analyzing responsible compounds for zone of inhibition. The quantitative analysis of the photochemical in leaves extracts showed thephotochemical constituents such as saponins, glycosides, flavonoids alkaloids and tannins, fixed oil, carbohydrate, reducing sugar,phytosterol, phenolic compound(Table:3)Alkaloids are toxic for microorganisms, hemolytic in nature and used as therapeutic agent in treatment of cancer (Soetan*et al*., 2009). It is also used for treatment Of renal disorder (Konkwara, 1976). Glycoside prevent the tumor growth an provide protection against gastro- intestinal infection (Adeshina, *et al*., 2010). Tannins have been reported as protein precipitating agents of microorganism (Sodipo

et al., 1991)Flavanoids, phenols and saponin have been reported for their antioxidative action and inhibitory effect on inflammation (Olayinka *et al*., 2010) Fixed oils and flavonoids have antimicrobial characteristics. The secondary metabolites identified in the plant leaves used in this study could be responsible for antimicrobial activity exhibited by these plants.

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