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ANTIBACTERIAL ACTIVITY OF PHYTO-CHEMICALS OBTAINED FROM LEAF-EXTRACTS OF SOME MEDICINAL PLANTS ON PATHOGENS OF SEMI-ARID SOIL

REKHA KHANDAL, SHILPENDRA KOUR AND RASHMI TRIPATHI
Department of Bioscience and Biotechnology, Banasthali University, Jaipur-304022, Rajasthan.
Email id: tripathi.rashmi@gmail.com
Address for Correspondence:- Dr.Rashmi Tripathi, Assistant Professor,
Department of Bioscience and Biotechnology, Banasthali University Banasthali – 304022. Rajasthan.
Mobile no: 9887566713 Email id: tripathi.rashmi@gmail.com

ABSTRACT

Plants being the major source of natural products like vitamins, minerals, immune–modulators, possess anti-pathogenic activities and act as good source of antimicrobial agents. This makes plants to occupy importance in traditional medicine. The phytochemical analysis revealed the presence of metabolites such as saponin, flavonoid, alkaloid, carbohydrate, glycoside, tannin and oil phenolic compound responsible for the antibacterial activity of these plants. Diffusion and dilution methods have been, employed to study the comparative antimicrobial activities of extracts of plants Neem (*Azadirachta indica*) Tulsi (*Ocimum sanctum*) and *Aloe vera*. The extracts of these plants under study, were used against bacteria isolated from the semi-arid soil in Banasthali region, the strains identified are *Escherichia coli*, *Pseudomonas fluorescence*, Bacillus *subtilis*, *Bacillus cereus* and *Staphylococcus aureus*. *Azadirachta indica* showed maximum inhibition against *S.aureus*, whereas *Ocimum sanctum* showed maximum zone against *E.coli* and *Aloe vera* against *Pseudomonas fluroscens*. The aqueous extracts of various leaves were investigated individually for antimicrobial activity against these pathogens and showed less inhibitory activity against most of the test bacterial pathogens.

KEY WORDS: Medicinal plant, Muller–Hinton agar, phytochemical analysis and antibacterial activity.

INTRODUCTION

In the traditional system of medicine plants play a very important role. Plants being rich in number of secondary metabolites saponin, alkaloids, flavonoids, steroids, tannins, glycosides, phytosterol and phenolic compounds are identified for the study of their antimicrobial potential and can be used for the treatment against a number of diseases (Nimri *et al.*, 1999; Saxena, 1997; Banso & Adeyemo *et al.*, 2006; Robe & Vanstrden, 1997; Ongaskul *et al.*, 2009; Ansan *et al.*, 2009). Drugs purified from the medicinal plants have been found to be very effective against many antibacterial, antifungal, antithrombotic, anti-malarial diseases and have been effective as anticancerous drug (Kaushik and Dhiman, 2000).The use of plant extracts as antimicrobial drugs is becoming very popular and significant, as microbes resistance to the drugs increased rapidly (Banso & Adeyemo *et al.*, 2006; Elbashiti *et al.*, 2010). The plants are very effective against treatment of many infectious diseases and the combination of the secondary metabolites is responsible for the physiological action on the body (Joshi *et al.*, 2009; Mishra & Mishra, 2011).

Neem (*A. indica*) has a vast array of biologically active compounds that are chemically diverse in nature. It has been found to be very effective antibacterial, antimalarial, contraceptive and antiulcer activities (Khan *et al.*, 2010; Bhuiyan *et al.*, 1997; Siddiqui *et al.*, 1992; Jones *et al.*, 1994).As the name Tulsi (*O. sanctum*) used as an expectorant, diaphoretic, anticancerous, anthelminthic, antiseptic, analgesic, antidiabetic and tonic rejuvenator. The oil extracted from the leaves of the plant is used in the treatment of number of diseases like bronchitis, skin diseases and otitis media. The leaf extracts of Tulsi plants have been found to be effective antibacterial and antifungal activity against number of strains (Nanasombat & Lohasupthawee, 2005; Hammer *et al.*, 1999; Goyal & Kaushik, 2011).*Aloe vera* is an ornamental and medicinal plant used against stomach disorders and intestinal disorders including constipation, colitis and colon problems. It is said to be natural cleaner, during the inflammatory process (Obata, 1993; Shelton, 1991).

Our plant resources have a great potential to be used as antimicrobial drugs and are widely being used worldwide against number of microbial diseases (Sofowora, 1986; Murugesan *et al.*, 2011). Large number of drugs prescribed today contains plant derived active bioactive compound and have been used for centuries as remedy for human diseases because they contain

Rekha Khandal and Shilpendra Kaurcompleted MSc. AMBT scoring good percentage from Banasthali University are preparing for NET examination and wants to pursue research in the field of Microbiology. They both are very good researchers and have a good knowledge of their subject.

Dr.Rashmi Tripathi did her Ph.D. in the field of aquatic toxicology from Lucknow University and joined Banasthali University as Assistant Professor in 2010. She is engaged in taking classes at both PG and UG levels of both Biotechnology and Biosciences and plans to pursue research in Aquatic Toxicology. She has several research papers to her credit in journals of repute and presented papers at international and national conferences. She has also received several awards for Presentation of her research papers and has guided seven students for dissertation under her supervision.

components of therapeutic value and are very diverse in molecular structure (Kaushik, 1985; Kohen & Carter, 2005). 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; Jhonson *et al*., 2011; Goyal & Kaushik, 2011; Singh & Kumar, 2012).

The present study was taken up to study the comparative antibacterial activity of the medicinal plants *A. indica, O. sanctum* and *Aloe vera* using different extracts against the bacterial pathogens *E.coli, P. fluorescence, Bacillus subtilis, Bacillus cereus and Staphylococcus aureus* extracted from the semi-arid soil. Phytochemical screening of the extract was, carried out to assess the presence of different phytochemicals in different extracts.

MATERIALS AND METHODS

The test organisms were isolated from soil collected from Krishi Vigyan Kendra Banasthali, Rajasthan. They were isolated and identified based on morphological and biochemical confirmation tests (Plate: 1: Fig 1 to 5). The identified isolated strains were *E.coli, B.subtilis, B. cereus, S.aureus, and P. fluorescence*. The fresh leaves of medicinal plants *A. indica, O. sanctum* and *A. vera* were collected, 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 powder was, further subjected for different extraction protocols.

Aqueous extraction

5g of air-dried powder of respective plant (*A. indica, O. sanctum, A. vera*) part viz., leaves, was boiled in 200 ml distilled water till one fourth of the extract initially taken was left behind after evaporation. The solution was, filtered using muslin cloth. Filtrate was, centrifuged at 5000 rpm for 15 min. The supernatant was again filtered using whatmann No.1 paper under strict aseptic conditions and the filtrate collected in fresh sterilized bottles and stored at 4°C until further use (Goyal *et al*., 2012).

Organic solvent extraction

For *Azadirachta indica* 10 g of leaf powder thoroughly mixed with 100 ml organic solvent (Methanol, Hexane, Chloroform and Petroleum ether) was take. The mixture obtained filtered through muslin cloth, re-filtered by passing through whatmann No.1 paper was collected. 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 final concentration 100mg/ml. each solution was stored at 4°C after collecting in sterilized glass tubes until use (Bhuiyan *et al*., 1997; Goyal *et al*., 2012)

For *Tulsi*, 10g was, thoroughly mixed with 100ml organic solvent (ethanol, methanol, hexane and ethyl acetate). Mixture was, placed at room temperature for 24h on shaker with 150 rpm. Solution was then filtered through muslin cloth and then re-filtered through whatmann No.1 paper the filtrate thus obtained was concentrated by complete evaporation of solvent at room temperature to yield the pure extract, each was stored at 4°c after collecting in sterilized bottles until further use. (Goyal *et al*., 2012; Mishra & Mishra, 2011)

For *Aloe vera* the plant gel collected, freezed dried and then grinded to get crude extract. The crude extract, filtered through whatmann No.1 paper. After overnight incubation at room temperature, the mixture, filtered, evaporated powder resuspended in organic solvent (ethanol, ethyl acetate, petroleum ether).

1 ml of organic extracts of all the plants taken in an eppendorf tube, subjected on rotatory evaporator at 4°c. A dry crude extract obtained, dissolved in sterile distilled water (Ahmad *et al*., 1998; Lin *et al*., 1999; Thiruppathi *et al*., 2010; Goyal *et al*., 2012) and used to study the antibacterial activity against the bacterial strains isolated.

Antibacterial activity:

Disc diffusion assay was, performed for antimicrobial screening (Andrews, 2000). Antibacterial activity potential against both Gram-positive and Gram-negative bacteria isolated from the soil. The disc (5mm) prepared from the whatmann No.1 paper and stored at 4°C in dark place, used for evaluating the antimicrobial activity. The antimicrobial activity of *A. indica, O. sanctum, A. vera* plant extracted with different organic solvents and aqueous extract is determined by disc diffusion method. Muller-Hinton agar was prepared and autoclaved121°C and dispensed into the sterile petriplates in aseptic conditions, allowed to solidify and bacterial culture was spread. The disc prepared by dipping in the plant extract and air-drying. The air-dried discs were then placed on the petriplates and plates incubated at 37° C for 24 hours. After incubation, the

growth of the zone of inhibition observed and antibacterial activity of the plant extract evaluated (Plate 2: Figures 6 to 10; Plate 3: Figures 11 to 15; Plate 4: Figures 16 to 20)

Phytochemical screening of the extracts:

The phytochemical screening of the crude extract was carried out (Table: 3) in order to ascertain the presence of its secondary metabolites such as saponin, alkaloids, flavonoids, streroids, tannins, glycosides, reducing sugar, carbohydrate, fixed oil, phytosterol and phenolic compound using standard procedure (Harbone, 1973; Odeibiyi & Sofowora, 1978; Santhi & Swaminathan, 2011; Viji, 2011).

(A) Determination of Saponin

5ml of extract added to 5ml of distilled water, shaken vigorously and warmed for 2 minutes formation of layer of permanent foam indicates the presence of saponin.

(B) Determination of Tannins:

Few drops of 5% $FeCl_3$ solution was added to small quantity of methanolic extract drop wise and any change in color 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 color shows the presence of anthocyanin, 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 phytosterol. 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 phytosterol.

(G)Determination of Glycosides

Salkowski test for the detection of glycoside, 2ml each extract dissolved in 2ml chloroform. Then 2ml H_2SO_4 added, shaken and formation of reddish brown colour indicates the presence of glycosides.

(H)Determination of Carbohydrates

Small quantity of extract dissolved separately in 4ml of distilled water and filtered. The filtrate treated with 2 to 3 drops of 1% alcoholic á-napthol solution, 2ml of concentrated Sulphuric acid 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's reagent and warm. Appearance of brick red precipitate shows presence of reducing sugars.

(J)Determination of Alkaloids

Take 1gm of each extract in different test tubes and add 2 drops of HCl. Add Mayer's reagent (freshly prepared by dissolving a mixture of 1.36g mercuric chloride and of 5 g potassium iodide in 100 ml water drop by drop. A Creamy white precipitate/ turbidity obtained. Turbidity of the extract on addition of Mayer's reagent was, regarded as evidence for the presence of alkaloids in the extracts.

Extraction of protein:

Performed to test whether active principle is protein or not. We take 1ml of each aqueous extract, mixed with protein denaturing agent Tannic acid (1:1). Incubate it for 1h; centrifuge at 4° C at 1000 rpm for 15 min until protein precipitated. Supernatant is collected applied for Lowry's to check residual proteins. Disk diffusion assay is, applied from this supernatant on Muller-Hinton agar base plates. Incubate at 37° C and zone of inhibition observed (Lowry's *et al.*, 1951).

RESULT AND DISCUSSION

Antibacterial activity of the selected medicinal plant extracts was tested against *E.coli, P. fluorescence, B.subtilis, B.cereus and S.aureus.* Considerable antibacterial activity of the all organic solvents such as Hexane, Chloroform, Petroleum ether, Ethyl acetate, Methanol, Ethanol exhibited maximum antibacterial activity against the gram positive and gram negative bacteria. The *A. indica* exhibited maximum inhibition against *S.aureus* in all the four solvents ranging from 6.5±0.64 to 4.25±0.47 mm. minimum activity exhibited in petroleum ether 5±0.40 mm for *S.aureus* and no activity against other bacterial strain. In *A. vera* maximum zone observed against *P. fluorescence* 27.25±0.75 to 23.25±0.47 mm. *A. vera* extracted by Ethyl acetate showed maximum resistance to *B.subtilis* (40±0.40 mm), Ethanol extraction showed maximum resistance to *S. aureus* (10.25±0.47 mm) and Petroleum ether was resistant to *P. fluorescens* 23.25±0.47 mm. In O. *sanctum* maximum zone of inhibition observed against *E.coli* 20±0.40 to 17.25±0.47 mm. Ethyl acetate extract of *O. sanctum*, resistant to *E.coli* (20±0.40 mm) *and P. fluorescence* (16.75±0.75mm). *A. indica* showed good results for all the extracts used against *S.aureus*. and *O. sanctum* and *A. vera* against *P. fluorescence* of the two plants, *A. vera* showed good results against *P. fluorescence.* (Figures 26, 27 & 28)

The aqueous extract showed less antibacterial activity against most of the test bacterial pathogens. Extracts investigated showed antibacterial activity,

Table 1: COMPARATIVE STUDY FOR MEDICINAL PLANT AGAINST GRAM+VE & GRAM ⁻ VE

Plant	ZONE OF INHIBITION in mm (AV±SE)					
	GRAM+VE			GRAM-VE		
	Plant extract	*B.subtilis*	*B.cereus*	*S.aureus*	*E.coli*	*P.fluorescens*
1.Azadirachta indica	Methanol	20.25±0.75	11±0.91	6.5±0.64	20±0.40	15.25±0.47
	Hexane	NI	4.25±0.47	4.25±0.47	8.75±0.47	NI
	Chloroform	21±0.91	6±0.40	4.25±0.47	19±0.40	NI
	Petroleum ether	NI	NI	5±0.40	NI	NI
2.Ocimum.sanctum	Methanol	8±0.40	5.25±0.47	6.25±0.47	17.25±0.47	19.25±0.47
	Ethanol	NI	4.5±0.28	5.5±0.28	20.5±0.28	14.5±0.64
	Hexane	NI	5.75±0.47	5.25±0.25	16±0.40	16.5±0.64
	Ethyl acetate	NI	NI	6±0.4	20±0.40	16.75±0.75
3.Aloe vera	Ethyl acetate	40±0.40	9.25±0.47	21.25±0.47	NI	27.25±0.75
	Ethanol	NI	3±0.40	10.25±0.47	NI	26.25±0.47
	Petroleum ether	NI	4.25±0.25	NI	4.66±0.28	23.25±0.47

TABLE - 2: Exhibiting Zone of inhibition after protein denaturation.

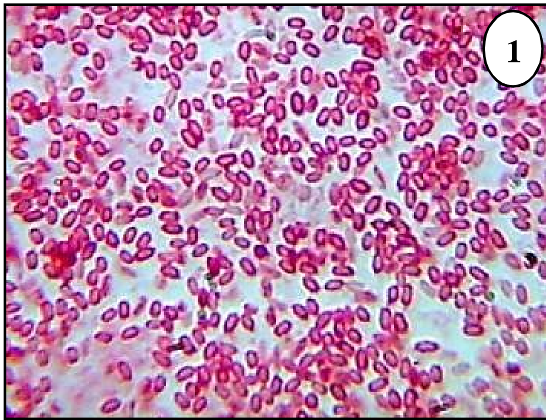
PLANT EXTRACT		PROTEIN (µg/ml)	ZONE OF INHIBITION (AV±SE)				
			B.subtilis	***B.cereus***	***S.aureus***	***E. coli***	***P.fluroscence***
1.Azadirachta.indica	**CRUDE Whole Protein**		**149.372**	**-**	**-**	**-**	**-**
	EXTRACT Denatured Protein	**61.632**	**11.75±0.25**	**14.25±0.47**	**11.5±0.28**	**10±0.40**	**13.75±0.47**
2.Ocimum.sanctum	**CRUDE**	**105.28**	**-**	**-**	**-**	**-**	**-**
	EXTRACT	**56.06**	**13.75±0.47**	**13.5±0.64**	**11.5±0.64**	**9.5±0.64**	**12±0.28**
3.Aloe vera	**CRUDE**	**214.856**	**-**	**-**	**-**	**-**	**-**
	EXTRACT	**77.04**	**12.25±0.47**	**13±0.40**	**8.25±0.47**	**12.25±0.47**	**13.5±0.28**

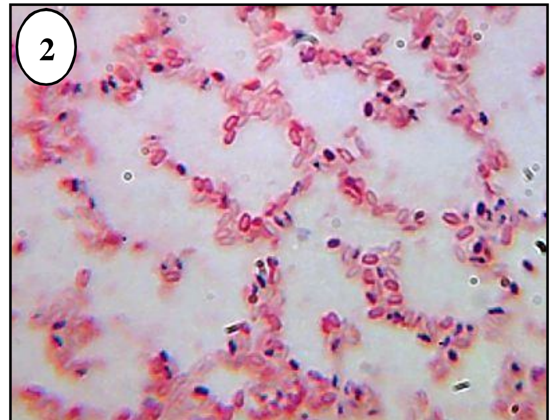
TABLE -3: PHYTOCHEMICAL ANALYSIS OF SOME MEDICINAL PLANT

S.NO.	PHYTOCHEMICAL TEST	*A. indica*	*O. sanctum*	*A. vera*
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	+	+	+

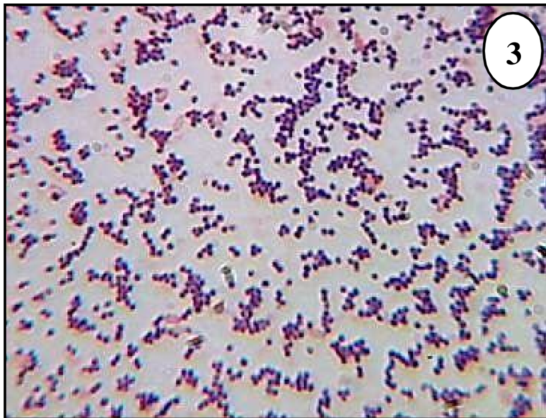
++ (Better Presence), + (Presence), - (Absence)

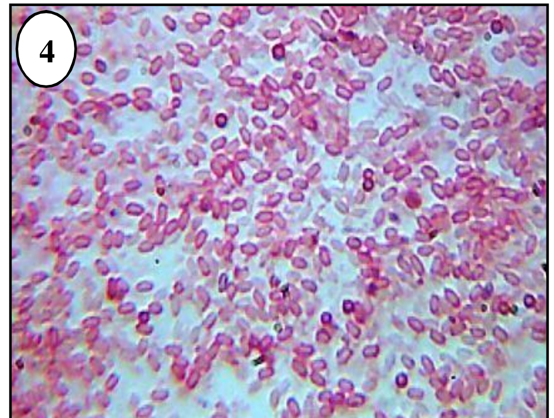
PLATE- 1

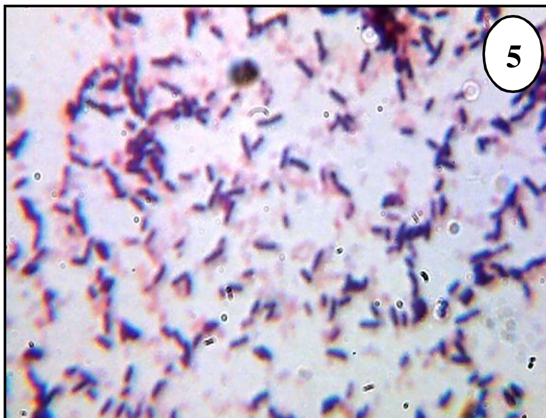


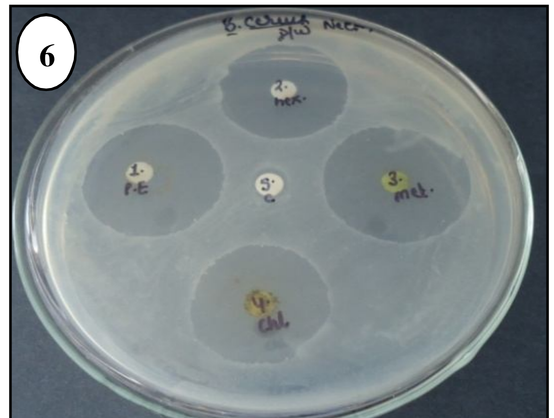


FIGURES 1 to 5 : Gram Staining of the Isolated Bacterial Strains.
(1) ***E.coli***
(2) ***P. fluorescens***
(3) ***S.aureus***
(4) ***B.subtilis***
(5) ***B.cereus***

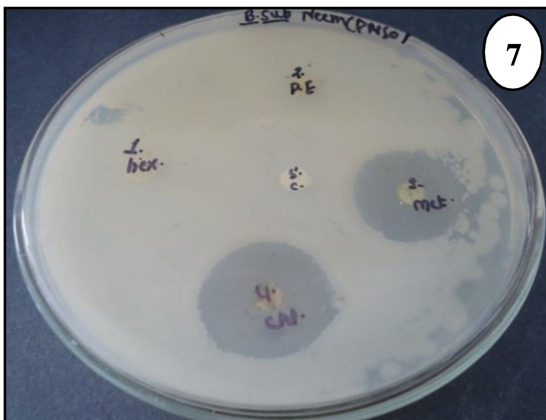


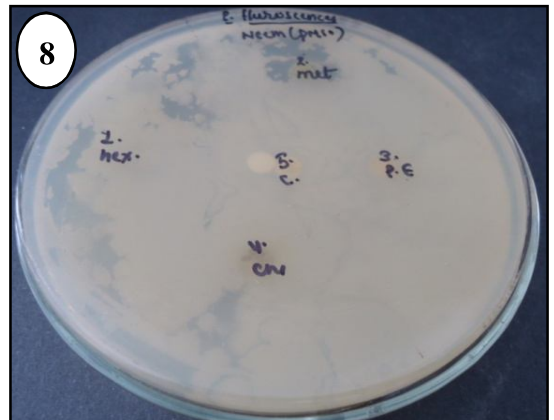


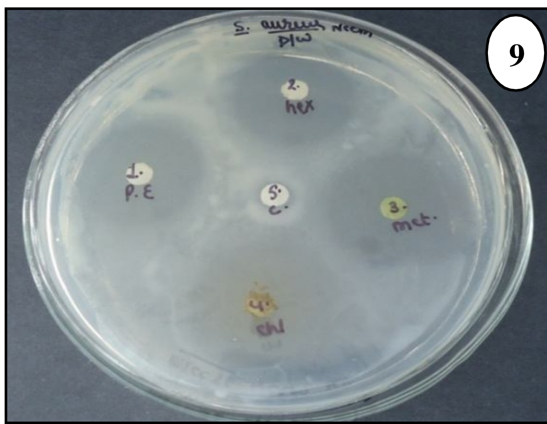


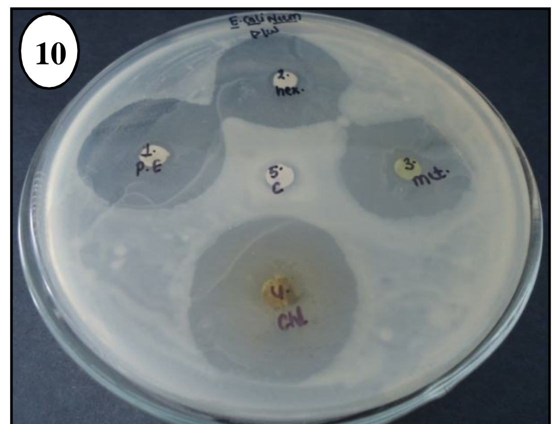


FIGURES 6 to10: Showing Antibacterial Activity of Plant Extract of ***Azadirachta indica*** **to isolated bacterial strains**
(6). ***B.cereus***
(7). ***B subtilis***
(8). ***P.fluorescens***

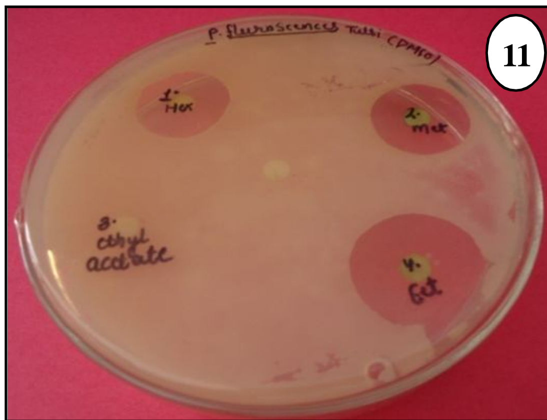


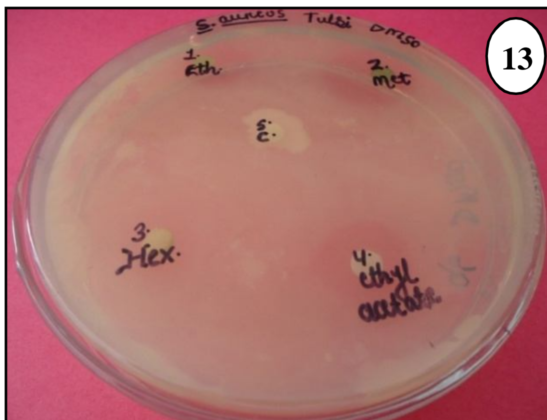




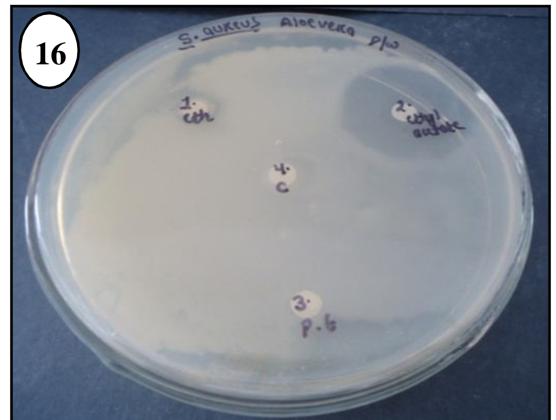


(9). *S.aureus*
(10). *E.coli*

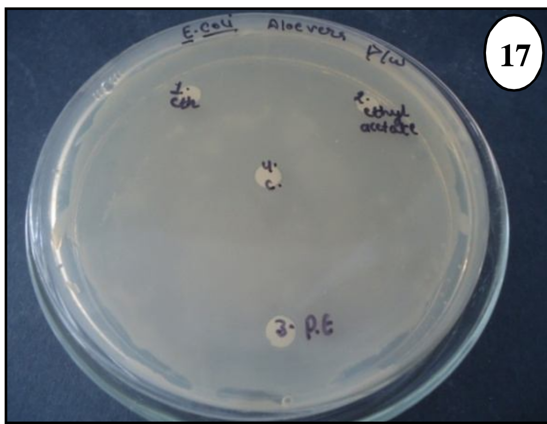


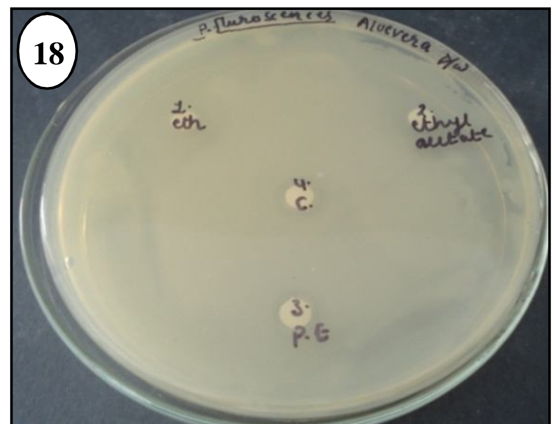


FIGURES 11 to 15: Showing Antibacterial Activity of Plant Extract of *Ocimum sanctum* to isolated bacterial strains
(11). *P.fluorescens*
(12). *B subtilis*
(13). *S.aureus*
(14). *B.cereus*
(15). *E.coli*

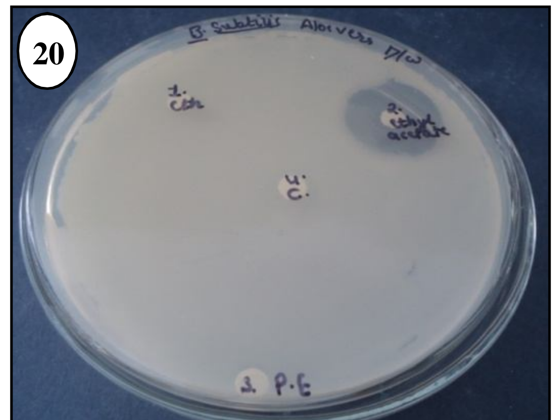


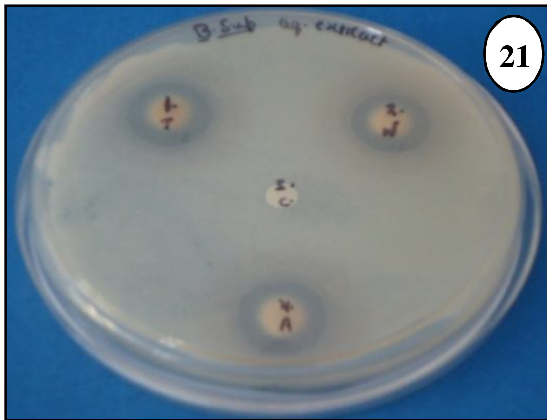
FIGURES 16 to 20 : Showing Antibacterial Activity of Plant Extract of *Aloe vera* to isolated bacterial strains
(16) *S. aureus*

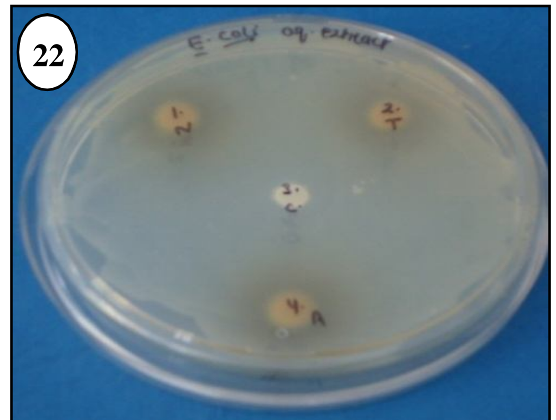




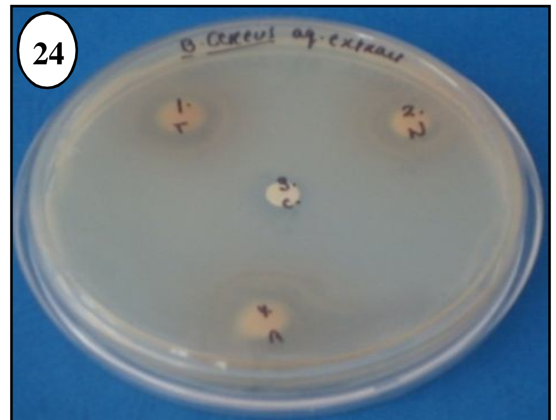
(17) *E.coli*
(18) *P.fluorescens*
(19).*B. cereus*
(20) *B subtilis*







FIGURES 21 to 25 :Showing Antibacterial Activity of Plant Aqueous Extract after Protein denaturation to isolated bacterial strains
(21). *B.subtilis*
(22) *E.coli*
(23).*P.fluorescens*
(24) *B. cereus*
(25) *S.aureus*



(25) *S.aureus*

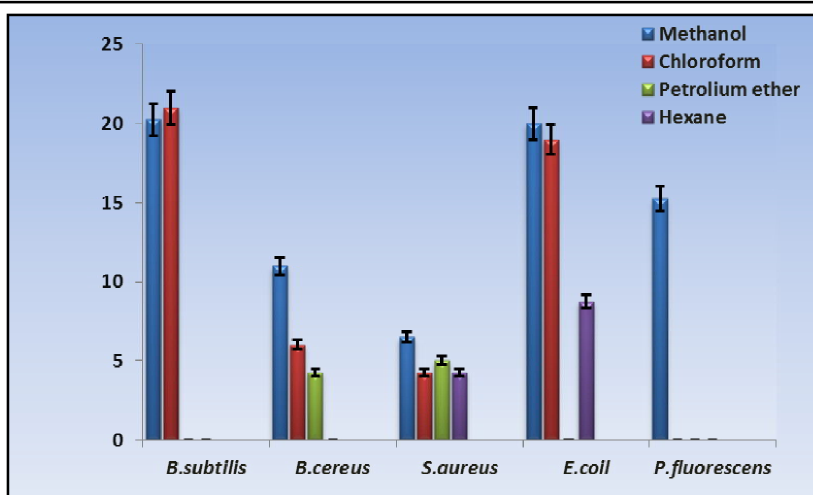


Figure 26: Antibacterial activity of *Azadirachta indica*

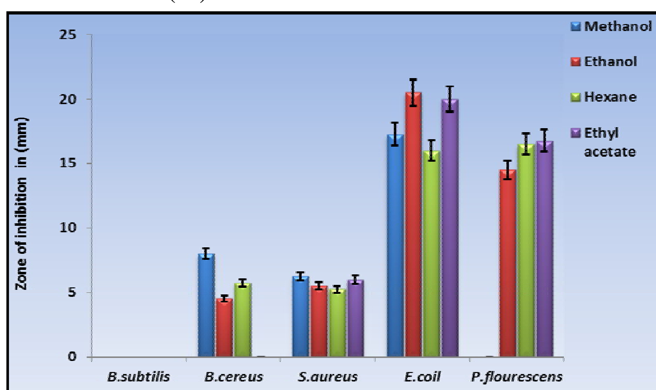


Figure 27: Antibacterial activity of *Ocimum sanctum.*

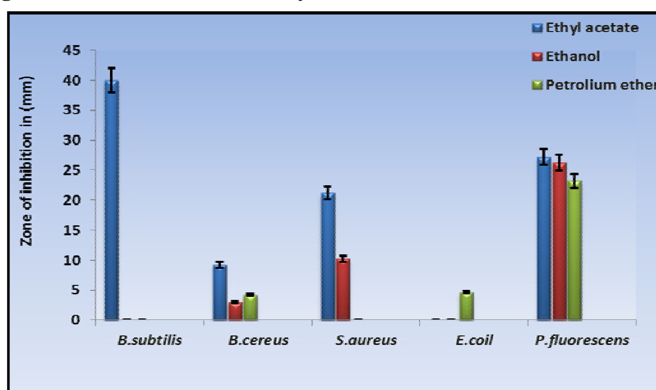


Figure 28: Antibacterial activity of *Aloe vera*

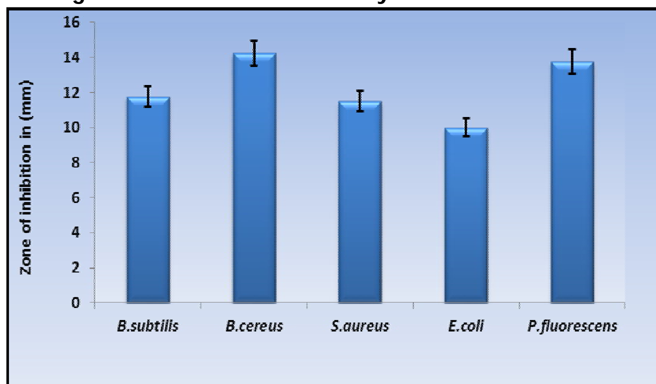


Figure 29: Antibacterial activity of aqueous extract of *Azadirachta indica* after protein denaturation

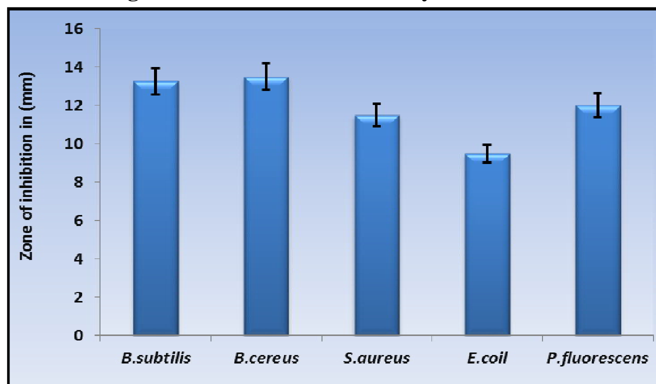


Figure 30: Antibacterial activity of aqueous extract of *Ocimum sanctum* after protein denaturation

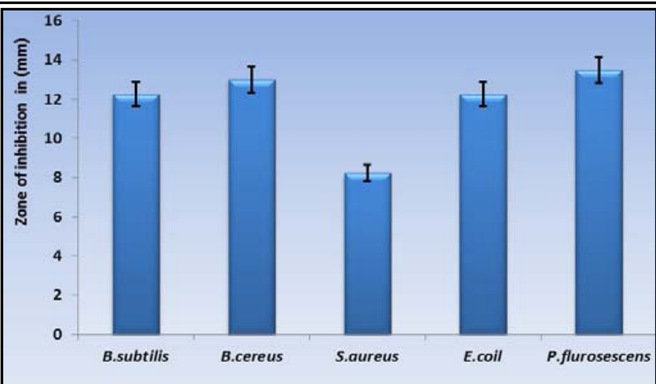


Figure 31: Antibacterial activity of aqueous extract of *Aloe vera* after protein denaturation

depends mainly on the solvents used for extraction. Organic solvents showed better results as compared to aqueous extracts but after protein denaturation, the aqueous extracts showed better results. The different susceptibility of bacteria to the plant extracts depends on the phytochemical constituents and the solvents used for extraction (Ntombeziningi, 2009). The bioactive compounds are responsible for the antimicrobial activity of plant extracts. Gram-positive bacteria is more susceptible than gram-negative bacteria, this is due to the difference in chemical composition and structure of cell wall of both type of microorganisms. The result of the phytochemical screening revealed the presence of

some active compounds saponin, glycosides, flavonoids alkaloids and tannins, fixed oil, carbohydrate, reducing sugar, phytosterol, and phenolic compound indicated in the (Table: 3) responsible for their antibacterial activity against the bacterial strains. Antibacterial activity due to bioactive compounds is been reported by many workers in plants (Freeman & Beattle, 2008; Xiao-tian & Wei-shuo, 2006). The aqueous extracts did not exhibit the inhibition zone. All the plant extract were evaluated for their protein content, the denatured extracts exhibited zones of inhibition, proves that protein is not the active bactericidal compound in the extracts(Plate 5: Figures 21 to 25; Table:2; Figure: 29 ; Figures 30 & 31). The present study also confirms the use of organic solvents in the preparation of plant extracts as compared to aqueous extracts. The polarity of antibacterial compounds makes them more readily extracted by organic solvents and it does not affect their antibacterial activity (Thongson *et al.*, 2004; Goyal *et al.*, 2012).

CONCLUSION

This study reveals that secondary metabolites are the possible antimicrobial substances. The present study also confirms the use of organic solvents to aqueous for extraction. The polar group of phytochemical compounds makes them more readily extracted by organic solvents, and using organic solvents does not negatively affect their bioactivity against bacterial strains (Owais *et al.*, 2005). It therefore suggests that constituents of the plant extracts could serve as a source of drugs useful against number of some microbial infections and diseases.

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