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Antimicrobial potential of *Berberis aristata* DC. against some human bacterial pathogens

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Berberis aristata DC (Family Berberidaceae) is one of the herbs mentioned in all ancient scriptures of ayurveda, Charakha and Susruta. Leaves of the plant were investigated for antimicrobial activity against six bacterial pathogens responsible for various ailments and infections in human body viz. *Staphylococcus aureus* MTCC7405, *Streptococcus epidermidis* MTCC3615, *Streptococcus mutans* MTCC890, *Escherichia coli* MTCC3221, *Klebsiella pneumoniae* MTCC7407 and *Bacillus cereus* MTCC7190 procured from Microbial Type Culture Collection Centre, Chandigarh, India. Plant extracts were made from powdered leaves by gradient extraction in the order of increasing polarity (hexane < chloroform < ethyl acetate < methanol < ethanol <water) using Soxhlet extractor.Agar -well diffusion method was employed to evaluate the zone of inhibition exhibited by individual plant extract against bacterial pathogens. Qualitative phytochemical analysis of the plant leaves was carried out to evaluate the presence of secondary metabolites. Active constituent was confirmed by GLC, TLC, HPLC and FTIR. Synergism between the active plant extract and few antibiotics was analyzed against three bacterial pathogens (*Staphylococcus aureus, Bacillus cereus* and *Klebsiella pneumoniae*).

Ethanol and methanol extracts exhibited maximum activities with zones of inhibition recorded 22 mm. and 15 mm. against *Staphylococcus aureus* and 12 mm. inhibition against *Klebsiella pneumoniae*. Ethyl acetate extracts profoundly inhibited *K. pneumoniae* (24 mm.) and *S. aureus* (18 mm.).Hexane and aqueous extracts moderately effected the growth of the pathogens, while chloroform extract was found weakly active. The leaves of the plant possessed three phytochemicals viz. tannins, saponines and steroides. Active constituent was isolated and confirmed as Berberin. Gentamicin presented significant synergism with active ethanol extract. Higher synergism was exhibited in case of S. *aureus* showing 28 mm. zone as compared to gentamicin alone (20 mm.), followed by *K. pneumoniae* (25 mm.), while plant extract with tetracycline combination recorded slight increase in zones of inhibition not establishing much synergism. Bacitracin along with active plant extract could not established effective synergism. The study may serve for the development of lead molecules and for the purpose of use of specific extracts in specific herbal formulations.

Key words: *Barberis aristata,* antimicobial potential, human bacterial pathogen

INTRODUCTION

Emerging and re-emerging zoonotic diseases, food-borne and water-borne diseases and diseases caused by multidrug resistant organisms constitute the major threats in India (Chugh, 2008). *Staphylococcus* and *Streptococcus* cause skin infections, meningitis, pneumonia and even overwhelming sepsis, a systemic inflammatory response producing shock, massive vasodilatation

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and death (Fish, 2002). Pathogenic variants of *Escherichia coli* cause intestinal and extra intestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis and septicemia (Von and Marre, 2005; Sodha *et al.*, 2011). *Klebsiella pneumoniae* is a successful opportunistic pathogen and has been associated with various ailments such as urinary tract infection, septicemia, respiratory tract infection and diarrhea (Podschun and Ullmann, 1998). *Bacillus cereus* produces a variety of toxins and cause serious infections such as poisoning, pneumonia, septicemia, central nervous system infection and endocarditic (Drobniewski, 1993; Bottone, 2010). In particular biofilm forming strains of *Staphylococcus epidermidis* that are also methicillin-resistant (MRSE) have become a very serious clinical problem as these infections are especially difficult to eradicate from colonized devices (Fitzpatrick *et al.*,2005)

In recent years attempts have been made to develop novel approaches to the screening of medicinal plants and other natural and synthetic compounds against MDR (multidrug resistant) bacteria, including screening for bioactive compounds/ plant extracts for (1) MDR efflux pump inhibitors, (2) α-lactamase inhibitors (3) synergistic approaches such as antibiotic-phytocompound synergy and (4) targeting virulence and pathogenicity of bacteria and use of quorum - sensing inhibitors (Usha *et al.*, 2008). Recent progress made in this direction indicated that the careful screening of potential plants and other natural products might provide novel compounds against MDR bacteria (Hassan *et al.*, 2011).

Some promising plants having antimicrobial activity against multidrug resistant strains have been reported by different workers *viz.*, *Allium sativum* against XDR (extreme drug resistant)- *Mycobacterium tuberculosis* (Hannan *et al.*, 2011),*Centratheram punctatum* against MDR-*Acinetobacter baumanni* (Pawar and Arumugam 2011), *Cinnamomum cassia* against MDR-*Pseudomonas aeruginosa* (Sharma *et al.* 2009), *Garcinia mangostana* against Vancomycin-resistant *enterococci* (VRE) (Sakagami *et al.*, 2005), *Psidium guajava* against MDR *Staphylococcus aureus* (Anas *et al.*, 2008), *Prosopis juliflora* against MDR *Acinetobacter* (Singh *et al.*, 2011) and *Thonningia sanguine* against ESBL-producing *Escherichia coli* (N'guessan *et al.*, 2007).

Berberis aristata DC. (Family Berberidaceae; Fig. 1) is one of the herbs mentioned in all ancient scriptures of ayurveda, Charakha and Susruta. It is an erect glabrous, spinescent, deciduous shrub, 3-6 m in height (Sharma *et al.*, 2002) with obovate to elliptic, subcute to obtuse, entire or toothed leaves, yellow flowers in corymbose racemes and oblong-ovoid to ovoid, bright red berries found in Nepal, grown in Nilgiris at an altitude of 1000-2400 m and all our temperate Himalayas at an altitude of 1000-3000 m (Anon, 2005). It is well known for its anti-inflammatory property (Gupta *et al.* 2008). The dried extracts of the roots are applied externally to eyelids to cure ophthalmic and other eye diseases. It is also reported to be a mild laxative, a tonic and is useful in curing ulcers and fevers (Kirtikar and Basu., 1993). The chief constituent of *B. aristata* DC. is berberine, which is bitter alkaloid (Rout *et al.*, 2008). The principle activity of this plant is due to the presence of berberine , oxycanthin , berbamine, and palmatine (Musumeci *et al.*, 2003) among which berberine exhibits multiple pharmacological activities (Fang *et al.*, 2004). It also has antibacterial, anti amoebic, antifungal, antihelminthic, leishmanicidal, and tuberculostatic properties (Soffar *et al.*, 2001).

There are various reports on antimicrobial activity of stem , root , bark and leaf extracts of *Berberis aristata* against different bacterial pathogens (Soffar *et al.*, 2001; Singh and Srivastava, 2007; Sasikumar *et al.*, 2007; Rout *et al.*, 2008 ; Gupta *et al.*, 2008 ; Shahid *et al.*, 2009 and Ahmad *et al.*, 2012) but this is the first holistic approach towards the study.

MATERIALS AND METHODS

The antimicrobial potential of *Berberis aristata* D.C. was tested against six bacterial pathogens viz. *Bacillus cereus* MTCC 7190, *Escherichia coli* MTCC 3221, *Staphylococcus aureus* MTCC 7405, *Streptococcus epidermidis* MTCC 3615, *Streptococcus mutans* MTCC 890 and *Klebsiella pneumoniae* MTCC 7407. These strains were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, and were maintained on nutrients-agar slants at 4°C and sub cultured for 24 h before use.

The powdered leaves, in different solvents were extracted by gradient extraction in the order of increasing polarity; hexane < chloroform < ethyl acetate < methanol < ethanol <water using Soxhlet

extractor (Jension, 2007). Sample of 200 g each was used for the extraction with 500 ml of the respective solvent .Each solvent was recycled for 6-7 h with the same batch. After extraction, the solvent was evaporated by means of rotary evaporator and weight of the dried material was taken. Qualitative phytochemical analysis was performed to detect the presence of various phytoconstituents in them (Raaman, 2006).

Antibacterial activity was measured using well diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993). Muller Hinton Agar was used for testing the antibacterial activity which was determined by zones of inhibition. The extracts showing antibacterial activity were assayed for Minimum Inhibitory Concentration (MIC) by micro dilution method using different concentrations of the extract solution *viz.*, 100 mg/ml, 70 mg /ml, 30 mg /ml, 10 mg/ml, 1 mg/ml and 0.5 mg/ml. in 1% dimethyl sulfoxide. Antibiotic sensitivity of bacterial strains were determined by well diffusion method on Muller Hinton agar against a number of standard antibiotics disc such as amoxicillin chloramphenicol, cephotaxime, gentamycin, kanamycin, rifampicin, tetracycline and bacitracin.

The antibacterial fraction (ethanol extract) showing highest antimicrobial activity was chromatographed on 2 × 30 cm silica gel 60 open column using a stepwise gradient of n-hexane and increasing amount of ethyl acetate (20 % at each step); ethyl acetate with increasing amount of methanol (10 % at each step); and finally at 40% methanol. Collected fractions were evaporated under vacuum and examined by TLC. Homogeneous fractions were pooled to give large no of different yellow colored crystalline fractions. Fractions were examined using Silica gel coated TLC plates to confirm the pure compound by changing the ratios of the solvent system components. The plates were developed in the solvent system to separate the compounds. Chromatograms were introduced into the iodine chamber for the observation of the compound. The active pure compound was scraped from the silica gel plate and eluted from the silica gel with ethanol. The active compound was filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica gel and this yielded more of compound fractions.

Identification of compound was done by using a combination of different techniques including HPLC and FTIR .Besides those characterization techniques, Rf values and melting point of the active compound were also determined .Synergistic activity of potent plant extract with antibiotics was evaluated by using well diffusion method. Synergism effect was considered when combinations exhibited with enlargement of combined inhibition zone size by 0.5 mm.

RESULTS AND DISCUSSION

Findings of qualitative estimation of different phytochemicals revealed that the plant material possesses saponins, tannins and steroids but in a previous study conducted by Ahmad *et al.* (2012) on stem and bark methanol extracts of *Berberis aristata,* the presence of alkaloids, glycosides, phenolic compounds, carbohydrates, flavonoids and sterols were reported.

It was found that among the six bacterial pathogens, *Staphylococcus aureus* was most susceptible against six different solvent extracts of *B. aristata* with maximum inhibition (22 mm) by ethanol extract, followed by ethyl acetate (18 mm) and methanol (15 mm). *Streptococcus epidermidis* was found to be most sensitive to ethanol extract with zone of inhibition of 11.5 mm. Though not all the solvent extracts could inhibit the growth of *Klebsiella pneumoniae* but the pathogen exhibited remarkable susceptibility against ethyl acetate extract of *Berberis* leaves with maximum zone of inhibition (24 mm) followed by 12 mm of zone diameter against both ethanol and methanol extracts of the plant. *B. cereus* was found susceptible to aqueous (11.5 mm), ethanol (10 mm) and methanol (10 mm.) extracts. *Escherichia coli* showed sensitivity against methanol, ethanol and hexane extracts producing (8 mm) zone of inhibition for each extracts. Pathogenic *Streptococcus mutans* was least sensitive among all six pathogens, the organism was found susceptible against ethanol and aqueous extracts with zone of inhibition (12.5 mm) and (6 mm) respectively (Table 1, Figs 2, 3 and 10).

MIC values of *B. aristata* leaf extracts against bacterial isolates were tested. *S. aureus* and *S. epidermidis* were found most sensitive against all the tested extracts with same MIC value of 10 mg /ml. On the contrary, *K. pneumoniae* was found susceptible only against ethanol extract with MIC

Table 1 : Antimicrobial activity of plant extracts against test pathogens

Pathogens	Et	Mt	Eta	Hx	Chl	Wat
	Zones of inhibition (mm)					
Staphylococcus aureus	22	15	18	12	8	12
Streptococcus epidermidis	11.5	10	10	10	NA	8
Streptococcus mutans	12.5	NA	NA	NA	NA	6
Bacillus cereus	10	10	NA	NA	NA	11.5
Klebsiella pneumoniae	12	12	24	NA	NA	NA
Escherichia coli	NA	8	8	8	NA	NA

Et: Ethanol, Mt: Methanol, Eta: Ethyl acetate, Hx: Hexane, Chl: Chloroform, Wat: Water, NA: No activity.

Table 2 : Minimum Inhibitory Concentration (MIC) of plant extracts against test pathogens

Pathogens	Et	Mt	Eta	Hx	Chl	Wat
	MIC (In mg /ml)					
Staphylococcus aureus	10	10	10	10	10	10
Streptococcus epidermidis	10	10	10	10	10	10
Streptococcus mutans	NIR	20	20	20	NIR	NIR
Bacillus cereus	10	20	NIR	NIR	NIR	10
Klebsiella pneumoniae	10	NIR	NIR	NIR	NIR	NIR
Escherichia coli	10	10	10	NIR	NIR	20

Et: Ethanol, Mt: Methanol, Eta: Ethyl acetate, Hx: Hexane, Chl: Chloroform, Wat: Water, NIR: No inhibition recorded

measured 10 mg/ml. *B.cereus* showed sensitivity against ethanol, methanol and aqueous extracts with MIC ranged 10 mg/ml to 20 mg/ml. *S. mutans* was found to be sensitive for methanol, ethyl acetate and hexane extracts with same MIC range of 20 mg/ml. *E. coli* was promisingly inhibited with MIC value of 10 mg/ml each against ethanol, methanol and hexane extracts. Ethanol and methanol extracts were profoundly active against most of the pathogens with MIC range limit between 10 mg/ml and 20 mg/ml (Table 2, Fig. 4 and 11).

Results of the present investigation are in correlation with the findings of Sharma *et al.*, (2011), which revealed that five solvent extracts (methanol, ethanol, acetone, hot and cold aqueous) of plant leaves showed great activity against *S. aureus, Pseudomonas mirabilis* and *P. aeruginosa*. Lately

Table 3 : Susceptibility / resistance of test pathogens to different antibiotics

Antibiotics	Zones of inhibition (in mm)					
	Sa	*Se*	*Sm*	*Bc*	*Kp*	*Ec*
Amoxicillin	20 S	20 R	15 R	15.6R	15 S	17 R
Cephotaxime	22 S	20.5R	20 R	18 R	25 S	27 S
Gentamicin	20 S	20 S	17 S	22 S	20 S	20.5S
Kanamycin	15.5 R	20 S	15 R	15 R	13 R	16.4R
Penicillin G	25.2 S	20 S	18.6R	14 R	-	-
Rifampicin	40 S	25 S	20 R	23.8S	8.6R	14 R
Tetracycline	30.2 S	25 S	20 S	27 S	20S	-
Chloramphenicol	18.6S	20 S	15.2R	25 S	20.5S	12 R
Ampicillin	20 S	22.4S	10.8R	14 R	17 R	14.2R
Ciprofloxacin	17.4R	20.5S	18 R	22 S	18.6S	15 R
Erythromycin	14 R	23.6S	19.3S	24 S	21 S	24.8S
Amikacin	23.8 S	27 S	12.8R	10.8R	15.2R	18.6S
Bacitracin	27 S	24.8S	15 R	28 S	18 S	19.5S

Sa: Staphylococcus aureus, Se: Streptococcus epidermidis, Sm: Streptococcus mutans, Bc: Bacillus cereus, Kp: Klebsiella pneumoniae, Ec: Escherichia coli

Table 4 : Synergism between active fraction and selected antibiotics

Pathogens	Synergistic zones of inhibition (mm)			
	Ext.	Gent.+Ext.	Tetr.+Ext.	Bacit.+Ext.
Staphylococcus aureus	22	28(20)	31(30)	23(27)
Bacillus cereus	10	24(22)	29(27)	27(28)
Klebsiella pneumoniae	12	25(20)	23(20)	18(18)

Ext: Extract; Gent: Gentamicin; Tetr: Tetracycline; Bacit: Bacitracin
A value in parenthesis pertains to zones of inhibition of standard antibiotics alone.

Gahlaut and Chhillar (2013) in their study on *B. aristata* leaves reported MIC of 5 mg/ml against *E. coli* towards aqueous extract. This significantly supports present findings on the effectiveness of plant extracts against *E. coli.*

Antibiotic sensitivity test for tested bacterial strains against a number of standard antibiotics such as

amoxicillin, chloramphonicol, cephotaxime, gentamicin, kanamycin, rifampicin, tetracycline and bacitracin revealed variability. Some strains found resistant to these antibiotics while some recorded sensitivity (Table 3).

Its melting point was found to be 182 °C. The pure compound was further subjected for antibacterial screening against the pathogens. The fraction was further subjected to HPLC and FTIR.

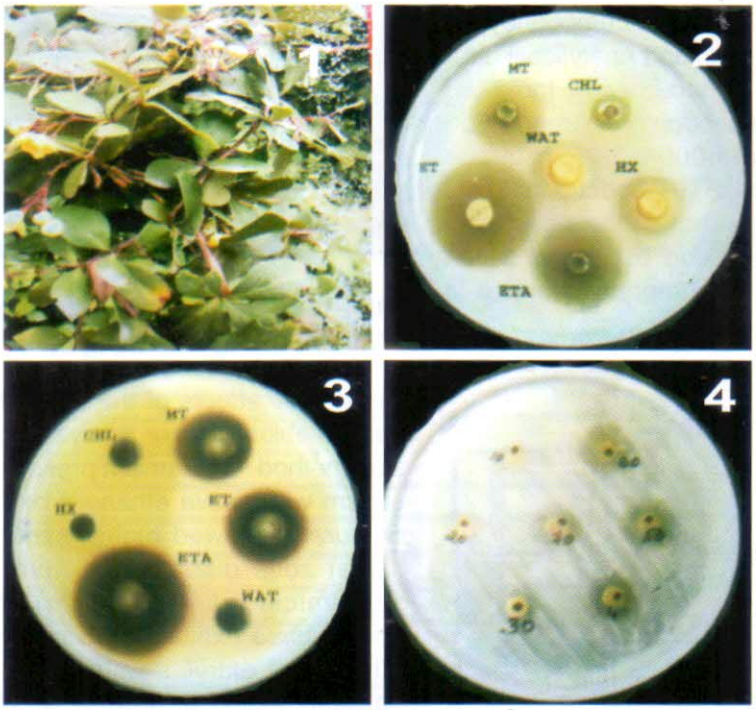

Fig. 1 : *Berberis aristata*
Fig. 2 : Antimicrobial activity of *Berberis aristata* against *Staphylococcus aureus*
Fig. 3 : Antimicrobial activity of *Berberis aristata* against *K. pneumonia*
Fig. 4 : MIC of ethanol extract against *Strcptococcus epidermidis*

Fig. 5 : TLC profile of pure isolated compound, Berberine from *B. aristata* (as observed as a single spot in iodine chamber) with reference to standard

The results of the pure compound separated on TLC are shown in Fig 5. The pure compound isolated was berberine chloride showing Rf value 0.56 similar to that of the standard compound berberine.

High Performance Liquid Chromatography (HPLC) was performed in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a Shimadzo LC 2010 HPLC system (Kyoto, Japan) equipped with a Shimadzo LC 2010 UV-VIS detector with a thermo stated flow cell and a selectable two wavelengths of 190-370 nm or 371-600 nm. The detector signal was recorded on a Shimadzo LC 2010 integrator. The column used was a C-18 block heating-type Shim pack VP-ODS (4.6 mm interior diameter 150 mm long) with a particular size of 5 mm. Mobile phase was designated as per the nature of the compound, containing 50 % acetonitrile along with 50% phosphate buffer at a flow rate of 3.0 ml /min, column temperature 25°C. Injection volume was 40 µl and detection was carried out at specific wavelength having maximum absorbance as calculated by UV absorption spectra at 254 nm. The retention time

of the pure compound purified was found to be 13.2 minutes as compared to the Standard reference compound. Berberine which showed its retention time 13.2 minutes while eluting out through the column. This analysis thus confirmed about the separation and identification of the purified active compound (Fig 6).

The Infra Red spectrum of isolated compound was recorded in Roorkee Research and Analytical Laboratory Pvt. Ltd. Roorkee (Uttarakhand), India using a computerized FTIR spectrometer (Perkin Co., Germany) in the range of 4000-5000 cm by the KBr pellet technique. The FTIR spectra peak of the compound merge with that of the spectra of reference /standard compound berberine which partially confirmed that the compound purified is

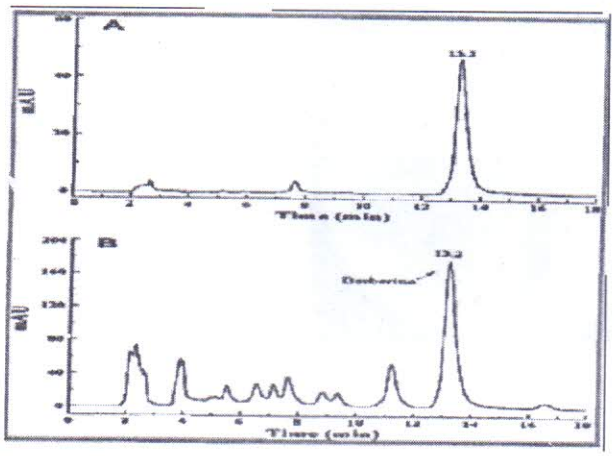

Fig. 6 : HPLC chromatogram of standard and berberine (isolated from *B. aristata*)

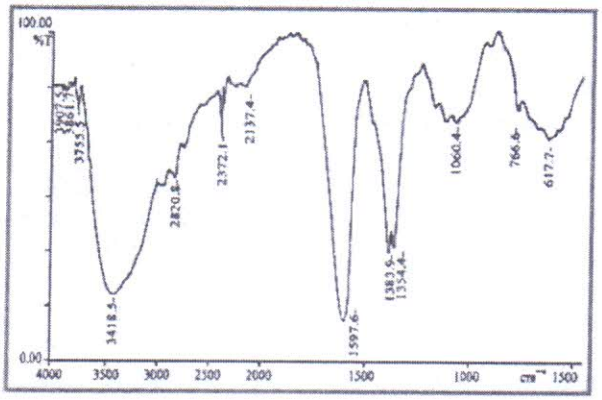

Fig. 7 : FT-IR spectra of isolated compound Berberine

having the similar nature to that of the standard. The C-H stretching was found at 2820 cm^{-1} while C=C and C=N stretching was observed at 1597 cm^{-1} .The deformation in C-H was found from

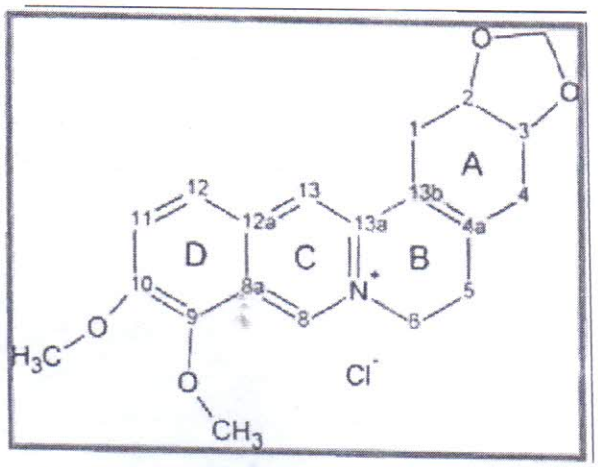

Fig. 8 : Structure of Berberine

1354-1383 cm^{-1} and C-O stretching was found at 1060 cm^{-1} (Fig 7 and Fig 8).

Synergistic effects of the active fraction with standard antibiotics were analyzed by agar well diffusion method. Gentamicin presented significant synergism with active ethanolic extract of *B. aristata*. *S. aureus* showed higher synergism , producing higher zone diameter (28 mm) as compared to gentamicin alone (20mm), followed by *K. pneumoniae* exhibiting 25 mm zone of inhibition. While plant extracts with tetracycline combination against tested pathogen recorded slightly increased zones of inhibition but these inhibition do not established synergistic effect. Bacitracin along with active fraction of the plant extracts could not established effective synergism (Table 4, Figs. 9 & 10).

Combined antibiotic therapy has been shown to delay the emergency of bacteria resistance and may also produce desirable synergistic effects in the treatment of bacterial infections. Drug synergism between known antibiotics and bioactive plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome).

Research on synergism is very limited and few studies have been reported using Kirby and Bauer method (Betoni *et al.*,2006) In the present findings it was found that synergistic effects of the antibiotics and plant extracts combination on tested bacterial pathogens is greater in terms of producing higher zones of inhibition as compared to plant extracts or antibiotics alone. Ethanolic extract of the plant along with tetracycline exhibited higher

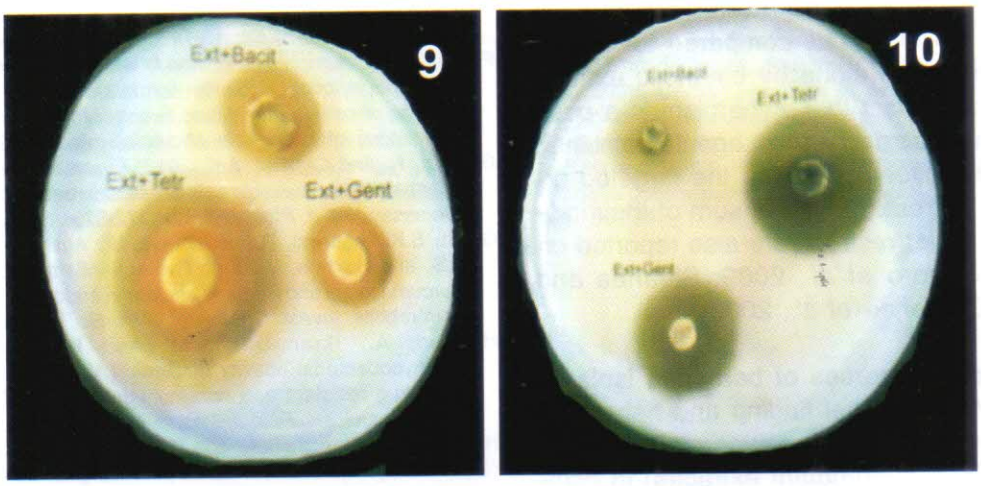

Fig. 9 : Synergism of antibiotics with ethanol extract against *Staphylococcus aureus*
Fig. 10 : Synergism of antibiotics with ethanol extract against *K. pneumonia*

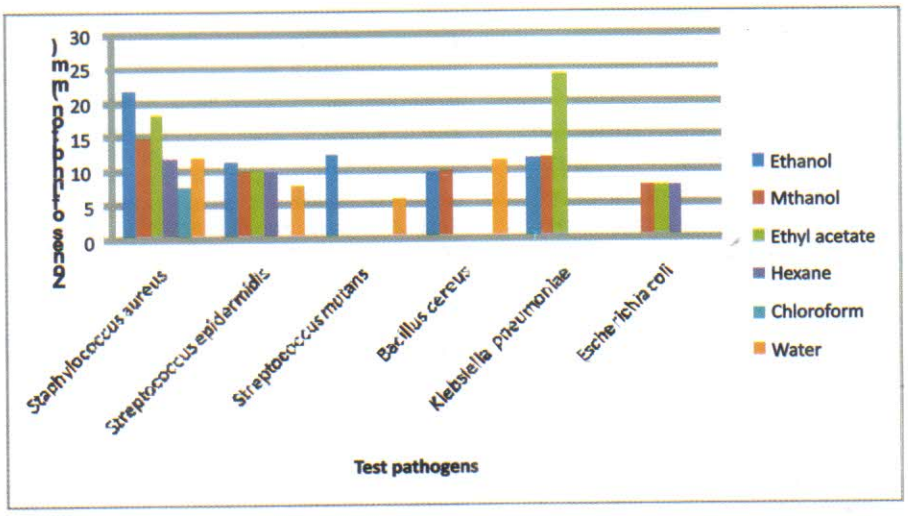

Fig. 11 : Antimicrobial activity of plant extracts against test pathogens

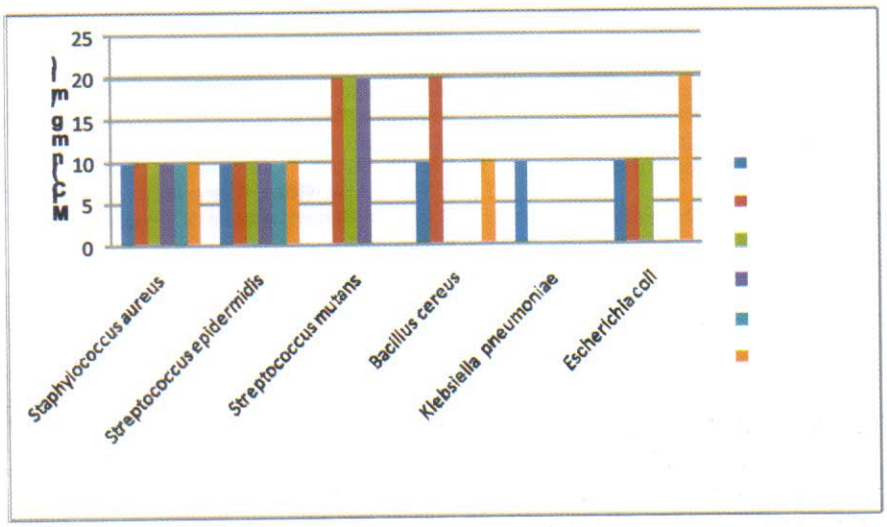

Fig. 12 : Minimum Inhibitory Concentration (MIC) of plant extracts against test pathogens

zones of inhibition (31 mm and 29 mm) against *S.aureus* and *B. cereus* as compared to 22 mm and 10 mm produced alone by ethanolic extract. This might be as a fact that, synergism is a positive interaction created when two agents combined and exert an inhibitory effect (on the targeted organisms) that is greater than the sum of their individual effects. Other researchers also reported on synergism (Aiyegoro *et al.*, 2009; Sibanda and Okoh, 2008; Chatterjee *et al.*, 2009).

The antibacterial properties of berberine isolated from *B. aristata* is a novel finding and hence important for drug resource. The synergistic interaction of plant bioactive(s)/potent extract(s) in combination with conventional antibiotics offers novel therapeutic leads to prevent resistance development and side effects of antibiotics and to effectively combat resistant infections.

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