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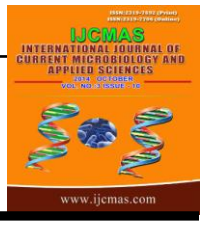

Original Research Article

In vitro Antibacterial Potency of *Butea monosperma Lam.* against 9 Clinically Isolated Multidrug Resistant Bacteria

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Keywords

Buteamonosperma, Gram-positive bacteria, Gram-negative bacteria, Multidrug resistant bacteria, Minimum inhibitory concentration, Antibacterial activity, Phytochemical constituents

A B S T R A C T

To investigate the antibacterial activity, using cold and hot extraction procedures with five solvents, petroleum ether, acetone, ethanol, methanol and water to validate medicinal uses of *Buteamonosperma* Lam (*B. monosperma*) in controlling infections; and to qualitatively estimate phytochemical constituents of leaf-extracts of the plant.The antibacterial activity of leaf-extracts was evaluated by the agar-well diffusion method against clinically isolated 9 Gram-positive and -negative multidrug resistant (MDR) pathogenic bacteria *in vitro*. Values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of leaf-extracts against each bacterium were obtained in a 96-well micro-titreplate, by broth dilution micro-titre plate technique.The presence of tannins, flavonoids, starch, glycosides and carbohydrates in different leaf extracts was established. Pathogenic bacteria used were, *Acinetobacter* sp., *Citrobacterfreundii*, *Escherichia coli*, *Klebsiellapneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus* sp., *Staphylococcus aureus* (*S. aureus*), methicillin resistant *S. aureus* and vancomycin resistant *S. aureus*, along with standard bacterial strains. These MDR bacteria had been recorded to have significant inhibitions by leaf extracts, obtained by cold and hot extraction procedures with five solvents. In addition, the hot aqueous extract against *Enterococcus* sp. had the highest inhibition zone-size (21 mm). Ciprofloxacin 30 µg/disc was the positive/reference control and the diluting solvent, 10% dimethyl sulphoxide was the negative control. Recorded MIC values of different extracts ranged between 0.23 and 13.30 mg/mL, and MBC values were 0.52 to 30.00 mg/mL, for these bacteria. Leaf-extracts with hot water and ethanol had shown significant antibacterial activity against all bacteria. *B. monosperma* leaf-extract could be used in treating infectious diseases, caused by the range of tested bacteria, as complementary and alternate medicine.

Introduction

Plant kingdom represents a rich source of organic compounds, many of which have been in use as agents against several infectious and non-infectious diseases, by the modern medicinal system.

The World Health Organization estimated that about an 80% population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs (WHO 2008, and Loeraa JA, *et*

al., 2007). Particularly in rural India, uses of raw plant products as well as some concoction of plant products in Ayurvedic medicines are sought after to a great proportion, because of cheap availability, and in urban areas too those are increasingly popular for cultural nuances that exist(De Silva T, *et al*., 2007). Further, a large number of phyto-drugs are popular and are preferred to over synthetic ones—*a priory*, for healthier or rather harmless effects (Sindhia VR, *et al*.,2010); almost all the viral infections are always addressed with plant products, as it is known. In ethno-botanical literature of India, several hundreds of plants are known to have the potential to treat many diseases and one of those popular ones is *Buteamonosperma* Lam. (*B. monosperma*, family Fabaceae (Kirtikar KR, *et al*., 1935).

B. monosperma is traditionally used for the treatment of inflammatory diseases (Shahavi VM,*et al*., 2008); it is hepatoprotective (Wagner H, *et al*., 1986), antidiabetic (Akhtar MS, *et al*., 2010), antihelmintic (Iqbal Z, *et al*.,2006), it possess antitumor, antiulcer activities and wound healing (Khan FM. 2009 andGavimath CC, *et al*., 2009), leaves possess antimicrobial property (Das S, *et al*., 2009, and Tambekar DH,*et al*.,2010), and roots have antispermatic activity (Vasudeva N, *et al*., 2011).

Infections with both Gram-positive (GP) and Gram-negative (GN) bacteria have clinically become intractable, slowly, due to the emergence of multidrug resistant (MDR) strains. Among GP pathogens, strains of *Staphylococcus aureus* (*S. aureus*), methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA), strains of *Enterococcus* sp. are noteworthy (Dubey D,*et al*., 2012). Moreover, GN bacteria, *Acinetobacter* sp., *Klebsiellapneumoniae* (*K. pneumoniae*), *Citrobacterfreundii* (*C. freundii*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are commonly found as pathogens of urinary tract; while *E. coli*, *K. pneumoniae*, are pathogens of gastrointestinal tract. Presently, these pathogens are too MDR, recorded in several reports (Dubey D,*et al*., 2012).

Secondly, the resistance of pathogenic bacteria to antibiotics is of high clinical concern. Rather the concept of the control of drug resistance is a matter of clairvoyance for dovetailed antimicrobials today. A suitable epitome is the superbug, multidrug resistant (MDR) *S. aureus* in the human health domain worldwide, as its different strains or rather incarnations have generated β-lactamase activities in degrading all sorts of penicillin derived antibiotics, in addition to resistance to other groups/generations of antibiotics (Clarke CR. 2006). Multidrug resistance of *Staphylococcus, Pseudomonas, Escherichia* and a few more pathogenic bacteria to a wide range of antibiotics has been reported to have been due to non-prudent uses of same antibiotics against infections of food- and pet-animals worldwide(Middleton J,*et al*., 2005). (Maple PAC, *etal*., 1989), including man. MDR-MRSA strains carry resistance markers for other antibiotics and instances of resistance up to 23 antibiotics in some strains have been reported(Maple PAC, *etal*., 1989). The emergence of VRSA is of further concern. Today, the management of the consortium of MDR strains of both GN and GP pathogens has become increasingly difficult because of the β-lactamase production by *Staphylococcus, Bacillus, Pseudomonas, Proteus, Klebsiella, Neisseria, Salmonella, Haemophilus* and a few more pathogens (Rath S,*et al*., 2012), and pandrug resistance (PDR, resistance of bacteria to all antibiotics in present use) to different classes of

antibiotics in GN ones (Falagas ME, *et al*., 1989). Meek appreciation of failures in the control of MDR strains would be inhuman, which generates the impetus on a systematic global search for new drugs from natural resources like plants, worldwide (Davidovich C, *et al*., 2008 andDubey D, *et al*., 2012); chemicals from plants could be chosen for the control in a future crusade against MDR pathogens. Moreover, accumulated ethnomedicinal reports of different countries lend themselves well to the basic information needed for further work on drug-targeting against MDR pathogens (Dubey D, *et al*., 2012).

In the present study, crude leaf extracts of *B. monosperma* with 5 solvents, petroleum ether, acetone, methanol, ethanol and water (polar to non-polar, extracted by both cold and hot extractions) were used to monitor antibacterial property against 9 clinically isolated MDR bacterial strains.

Materials and Methods

Preparation of plant extract

The air-dried powdered leaf material (in 40 g lots) of *B. monosperma* was extracted with 400 mL volumes of solvents, petroleum ether, acetone, methanol, ethanol and distilled water, separately at 4 °C, in succession. Solvent residues from combined extracts were evaporated by a vacuum rotary evaporator. For hot extraction, in a soxhlet apparatus, a lot of 40 g of powder-mass was placed in the extractor and a volume of 400 mL of a solvent was used during 24 h of soxhletion, till colourless extracts precipitated in the extractor. After filtration, each extract was concentrated by the rotary evaporator. The resultant sticky-mass was dried in a desiccator; the solid mass was stored in a suitable volume of 10% dimethyl sulphoxide (DMSO) with a drop of Tween-80. Cold and hot petroleum ether extracts of *B. monosperma* were light yellow to yellowish brown in colour. After concentration, the solid physical appearance was seen and the yield amounts were 3.7% in the cold- and 4.2% in the hot-extract. The solid extract was dissolved in a required volume of 10% DMSO and a drop of Tween-80 for a final concentration of 30 mg/mL. Both cold and hot extracts of acetone were brown in colour, it was sticky in appearance after concentration and the yield amounts were 3.12% in the cold and 4.27% in the hot extract. Ethanol extracts were reddish brown in colour and sticky after concentration. The yield amounts were 6.24% in the cold and 7.90% in the hot extract. Methanol extracts were dark-brown to black in colour and solid, sticky in concentration; after the desiccation amounts were 7.20% in the cold and 8.20% in the hot extracts. Aqueous extracts were black in colour and sticky after the concentration. After desiccation, the amounts were 10.78% in cold and 10.82% in hot extracts. The stock concentration of each extract was maintained at 30 mg/mL, for further use.

Qualitative test for phytochemicals

Phytochemical screening was carried out to assess the qualitative chemical composition of crude extracts using commonly employed precipitation and colouration procedure to identify the major natural chemical groups, as described earlier (Dubey D, *et al*., 2012). Alkaloids, carbohydrates, flavonoids, glycosides, protein, saponin, starch, sterols and tannins were assessed.

Isolation and identification of pathogenic bacteria

From hospitalized patients of wards and cabins of Hospital, 9 bacterial strains (four GP species, *S. aureus*, MRSA, VRSA and *Enterococcus* sp.; and eight GN bacteria, *Acinetobacter* sp., *K. pneumoniae*, *C.*

freundii, *E. coli* and *P. aeruginosa*) were isolated. All these 9 strains were identified by standard biochemical tests and were maintained as axenic cultures in suitable media, as described previously (Dubey D,*et al*., 2012 and Rath S, *et al*., 2012). Different clinical samples were collected from patients of wards, cabins, intensive care unit, neonatal care unit in the hospital, and were used for the growth of bacteria in nutrient agar, MacConkey agar, blood agar, eosin methylene blue (EMB) agar, and xylose lysine deoxycholate (XLD) agar. Microbial type culture collection (MTCC) strain of each bacterium was used as the reference control during identification (see Table 1).

Antibiotic sensitivity pattern

All bacterial strains were subjected to antibiotic sensitivity test by Kirby-Bauer's method, using a 6 mm thick Mueller-Hinton agar medium, as described previously (Sahu MC*et al*., 2012).and results were determined basing upon the standard guidelines (Clinical Laboratory Standard Institute Performance standard for antimicrobial susceptibility testing 2011). For the control, ciprofloxacin 30 µg/disc was used and it was sensitive to all test bacteria and its inhibition zone range was 17-19 mm.

Antibacterial activity and determination of MIC and MBC

Antibacterial activities of plant-extracts (both cold and hot) were recorded by the agar-well diffusion method, as described previously (Dubey D,*et al*., 2012 andDubey D, *et al*., 2012). The details of methods of determinations of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were described previously (Sahu M C, *et al*., 2012).

Result and Discussion

Isolation and biochemical identification of bacteria

Specific colony morphology of each pathogen was noted, for which a corresponding MTCC strain was used, parallely (Table 1). For example, colourless smooth, opaque, raised and pinpoint colonies were of *Acinetobacter* sp. After growth, a single colony was subjected to Gram-staining and basing upon it, other biochemical tests were performed for identification (Table 2). For example, *E. coli* was negative for oxidase, Voges-Proskauer, citrate and urease tests, while bile-esculin was not done; it was positive for catalase, indole, and methyl red, triple sugar iron and nitrate reduction tests. Similarly, the rest bacteria were typified. Four GPs, *S. aureus*, MRSA, VRSA, *Enterococcus* sp. and six GNs, *Acinetobacter* sp., *C. freundii*, *E. coli*, *K. pneumoniae*, *Proteus* sp., and *P. aeruginosa*, were isolated.

Phytochemical analyses

From phytochemical analyses, it was ascertained that saponins and tannin, but not sterol and protein were present in leaf-extracts, obtained with petroleum ether, acetone, ethanol, methanol and water. Alkaloids, carbohydrates, glycosides were present in extracts obtained with petroleum ether, ethanol and water. Starch only was found in extracts obtained with acetone and water; in water extract the maximum number of phyto-constituents and the acetone extract had the least number were noted, and extracts with the rest other solvents had medium levels of phyto-constituents (Table 3).

Antibiotic sensitivity pattern

Antibiotic profile of each bacterial strain was determined using specified antibiotic discs (Table 4).

It was recorded that antibiotics (µg/disc), gentamicin 30 was resistant to seven bacteria and sensitive to one bacteria; norfloxacin 300 was resistant to three and sensitive to five strains; nitrofurantoin 300 was resistant to one and sensitive to seven strains; amikacin 30 was resistant to one and sensitive to seven bacteria; cefotaxime 30 was resistant to seven; imipenem 10 was resistant to seven and sensitive to one; piperacillin/tazobactam 100/10 was resistant to seven and sensitive to one bacterium; gatifloxacin 30 was resistant to four and sensitive to four isolates; ofloxacin 5 was resistant to four and sensitive to four strains; netilmicin 30 was resistant to five and sensitive to three isolates; amoxyclav 30 was recorded as resistant to five and sensitive to three bacteria.

Antibacterial activities

Five pairs of cold and hot leaf-extracts extracted with petroleum ether, acetone, ethanol, methanol and water (non-polar to polar solvents) were screened for anti-bacterial activity against cited GP bacteria and five GN bacteria. Hot water extracts had the highest antibacterial activity against *Enterococcus* sp. (Table 5). Leaf-extracts with hot water and ethanol have shown significant antibacterial activity against all bacteria. the maximum size of zone of inhibition had been recorded due to the hot water extract, as 21 mm against *Enterococcus*, which was 18 mm in the case of ciprofloxacin 30 µg/disc. Detailed information of antibacterial activities of extracts and inhibition zone sizes were recorded (Table 5).

MIC and MBC values

Particular leaf-extracts obtained with different solvents that have shown significant antibacterial activity in the agar-well diffusion method were further used for the determination of MIC and MBC values with bacteria, in a 96-well micro-titre plate (Table 6). MIC values of all hot extracts ranged from 0.23 to 13.30 mg/mL, and MBC values ranged from 0.52 to 30.00 mg/mL. The MIC value of the cold acetone extract ranged between 0.52and 5.91 mg/mL, the MBC value ranged between 1.16 and 13.30 mg/mL; with hot ethanol extract, the MIC value ranged from 1.16 to 13.30 mg/mL, the MBC value ranged between 5.91 and 30.00 mg/mL; for methanolic extract the MIC values ranged between 1.16 and 5.91 mg/mL, the MBC value was 2.62 to 13.30 mg/mL; the MIC value with the cold water extract was 1.16-5.91 mg/mL and the MBC value ranged between 2.62 and 13.30 mg/mL.

With *Acinetobacter* sp., the minimum MIC in petroleum ether, methanol and water extracts was 2.62 mg/mL, the maximum MIC in petroleum ether extract was 13.30 mg/mL. With *Citrobacter* sp., the minimum MIC was with petroleum ether, acetone, and methanol extracts as 2.62 mg/mL, and the maximum MIC value was with ethanol and water extracts as 5.91 mg/mL. With *E. coli*, the lowest MIC was with methanolic extract as 5.91 mg/mL, and the highest MIC value with the ethanolic extract was 13.30 mg/mL. With *K. pneumoniae*, the lowest MIC was with the Methanolic extract as 1.16 mg/mL, and the highest MIC value was with the ethanolic extract as 5.91 mg/mL.With *P. aeruginosa*, the lowest MIC was with petroleum ether extract at 0.52 mg/mL, the highest MIC value was with water extract as 5.91 mg/mL. With *Enterococcus* sp.,

minimum MIC value was seen with the water extract as 0.23 mg/mL, and the maximum MIC value was with ethanol and methanol extract as 5.91 mg/mL. With *S. aureus*, the minimum MIC value was as 0.52 mg/mL and with the acetone extract, and the maximum MIC value was with the petroleum ether extract as 5.91 mg/mL. With MRSA, the minimum MIC value was with ethanolic extract 1.6 mg/mL, the maximum MIC value was as 5.91 mg/mL, due to ethanol, methanol and water extracts. With VRSA, the minimum MIC value was with the acetone and ethanol extracts as 1.16 mg/mL, and the maximum MIC value was with the methanol and water extracts as 5.91 mg/mL.

With *Acinetobacter* sp., minimum MBC was 5.91 mg/mL with the aqueous extract, and the maximum value with the petroleum-ether-extract was 30.00 mg/mL. With *Citrobacter* sp., the minimum MBC value was 1.16 mg/mL with the acetone extract, and the maximum value with petroleum-ether, ethanol and water extracts was 13.30 mg/mL.With *E. coli*, the minimum MBC value was 5.91 mg/mL in ethanolic extract, with the maximum value by ethanolic extract as 30.00 mg/mL.With *K. pneumoniae*, the minimum MBC value was 2.62 mg/mL in methanolic extract and the maximum value with petroleum-ether-extract was 30.00 mg/mL. With *P. aeruginosa*, the minimum MBC value with petroleum ether extract was 1.16 mg/mL, the maximum MBC value with water extract was 13.30 mg/mL.With *Enterococcus* sp. the minimum MBC value with water extract was 0.52 mg/mL, while the maximum MBC value with methanolic extract was 13.30 mg/mL. With *S. aureus*, the minimum MBC value with acetone extract was 1.16 mg/mL, while the maximum value with petroleum-ether extract was 13.30 mg/mL. With MRSA, the minimum MBC value with ethanolic extract was 2.62 mg/mL, the maximum value with water extract was 13.30 mg/mL. With VRSA, the minimum MBC value with acetone extract was 2.62 mg/mL, the maximum value with petroleum-ether extract was 30.00 mg/mL.

The presence of tannins, flavonoids, starch, glycosides and carbohydrates in leaf-extracts was established; saponins and tannins were present in all five-extract pairs, whereas sterols and proteins were absent in all extracts; alkaloids, carbohydrates, glycosides were present in three extracts, obtained with petroleum ether, ethanol and water, in this study. As reported, starch was found in leaf-extracts obtained with acetone and water only, whereas, flavones and flavanols (Yadava RN, *et al* ., 2009),chalcones (Gupta SR, *et al*., 1970), isoflavones, triterpenes and pterocarpans (Bandara BMR, *et al*., 1990), leucocyanidin tetramers (Seshadri TR, *et al* ., 1971), and sterols were recorded with *B. monosperma* flower-extracts (Mishra M, *et al*., 2000 and Chokchaisiri R, *et al* ., 2009); the presence of seven flavonoids and glycosides, with two of them (butrin and isobutrin) was recorded (Wagner H,*et al*.,1986). Three glycosides, coreopsin, isocoreopsin and sulfurein were identified, and the remaining two were new and had been assigned the structures—monospermoside and isomonospermoside(Chokchaisiri R, *et al*., 2009). Extracts of *B. monosperma* flowers registered the anticonvulsive activity, due to the presence of a triterpene(Kasture VS,*et al*., 2002).

It had been shown that *B. monosperma* exhibited antifungal activity (Singh V. 2011).Ethanolic bark extract had registered a good control on National Type Culture Collection bacterial strains (drug sensitive strains of *S. aureus*, *Bacillus cereus*, *P. aeruginosa* and *E. coli*), with the highest sizes of zones of inhibition against the used bacteria, at around 100 mg/mL with the

aqueous extract of the plant (Lohitha P, *et al*., 2011). Ethanolic extract of *B. monosperma* bark inhibited the growth of drug sensitive strains of *S. aureus, P. aeruginosa* and the fungus, *Candida. albicans* at 400 to 800 mg/mL (Sharma AK, *et al*.,2011). Antimicrobial activity of the bark extract with petroleum ether and ethanol in controlling *S. aureus*, *B. subtilis*, *S. typhi*murium and *E. coli* were the minimum (Gurav SS, *et al*., 2008).

In vitro control capacities of the aqueous extract of *B. monosperma* on *E. coli*, *S. aureus*, *Enterococcus*sp were significant (Tambekar DH, *et al*., 2010). Antimicrobial activities against extracts of *B. monosperma* using ethanol, chloroform, petroleum ether seen with *E. coli*,and *S. aureus*, were significant. The petroleum-ether-extract did not inhibit by any bacterium (Dhale DA, *et al*., 2010), which finding too corroborated this work. Moreover, wound-healing capacity of *B. monosperma* had been demonstrated (Gavimath CC, *et al*., 2009). Antimicrobial activities of the aqueous extract of the plant had been recorded against the destructive enteric pathogen, *Vibrio cholerae* at the level of 4 mg/mL, with the total gallic acid equivalent at 136 mg (Acharyya S, *et al*., 2009).

MRSA strains reported from Nepal were at 40.1% of the total bacterial isolates, and those strains were multiple resistant to trimethoprim/sulfamethoxazole, cephalexin, amikacin, ciprofloxacin and norfloxacin, in addition to the usual penicillin derivatives, but all those were vancomycin sensitive (Tiwari HK, *et al*., 2009). But the most effective way to prevent clinical crisis due to MRSA has been with daptomycin, nowadays (Holloway K. 2000 and Sorlozano A,*et al*.,2009). In Brazil, about 40% to 60% nosocomial infections in urinary and respiratory tracts, boils and surgical wound infections were by MRSA alone, and the presence of *mec*A gene with those was proved, probably because of such a greater infection prevalence (Perez LRR, *et al*., 2008). In a study from Malaysia, it was reported that among 287 pathogens, 52% were GNs with *Proteus* sp. 25%, *P. aeruginosa* 25%, *K. pneumoniae* 15%, *E. coli* 9%, and the rest 45% were GP bacteria with *S. aureus* 40%, Group B *Streptococci* 25% and *Enterococcus* sp. 9%; antibiograms indicated the susceptibility to imipenem and amikacin in GN and vancomycin in GP bacteria (Raja NS. 2007). Among intracellular pathogens isolated, both *S. aureus* and *Staphylococcus epidermidis* were frequently present, the latter species being coagulase-negative *Staphylococcus*; and *S. aureus* strains were mostly MRSA. Indeed, *S. aureus* was not invasive intrinsically, but MRSA was reported as invasive through eye (Kato T, *et al*., 1998). Further, in a classical study from New York, it was reported that the colonization rate of MRSA was more in intravenous drug abusers (Berman DS, *etal*., 1987).

While analysing the infection dynamics of pathogens, it was obvious that antibiotic sensitive pathogens have a limited capacity of virulence as the employed antibiotic controls them. At several levels, the host defence system also helps the control of pathogens when the later are in a smattering number. Most often than not, an infection from a MDR bacterial strain leads to a disease, particularly when an emulating control-agent/antimicrobial is absent, *i.e*., the employed antibiotic has been won over by it. Indeed, in the presence of a stress factor—an antibiotic, the bacterial cell undergoes intrinsic or acquired genetic changes via, conjugation/transformation, involving exchanges of resistance markers, exemplified with the *mar*-locus of *E. coli*(George AM, *et al*., 1983), if at least, the natural selection for the emergence of mutants is slow.

Table.1 Isolation and characterization of pathogenic clinical isolates with individual colony characteristics

Bacteria	Standard strain	Agar media	Colony morphology
Acinetobacter sp.	MTCC 1425	Nutrient agar	Colourless smooth, opaque, raised and pinpoint colonies
		MacConkey agar	Colourless smooth, opaque, raised, NLF colonies
C. freundii	MTCC 1658	MacConkey agar	Late LF colonies light pink after 48 h
E. coli	MTCC 443	Nutrient agar	Flat dry, irregular colonies
		MacConkey agar	LF, flat dry pink, irregular colonies
		EMB agar	Flat dry, irregular colonies, with metallic green colour
K. pneumoniae	MTCC 4031	MacConkey agar	LF, pink, mucoid colonies
P. aeruginosa	MTCC 1688	Nutrient agar	Large, irregular opaque colonies, with bluish green pigment
Enterococcus sp.	MTCC 439	Blood agar	Smooth, opaque, colourless colonies
S. aureus, MRSA, VRSA	MTCC 7443	Blood agar	Medium to large, smooth, entire, slightly raised, creamy yellow, with green/β hemolytic colonies
		Nutrient agar	As in blood agar without hemolytic activity

MRSA: methicillin resistant S. aureus; VRSA: vancomycin resistant S. aureus; LF: lactose fermenting colonies; NLF: non-lactose fermenting colonies; EMB: eosin methylene blue agar; NA: not available

Table.2 Summary of results of biochemical tests of nine pathogenic bacteria

Catalase	Oxidase	Indole	MR	VP	Citrate	Urease	TSI	NT	BE
+ve	-ve	nd	nd	nd	nd	nd	nd	-ve	nd
+ve	nd	nd	+ve	-ve	+ve	-ve	A/G	nd	nd
+ve	-ve	+ve	+ve	-ve	-ve	-ve	A/G	+ve	nd
+ve	-ve	-ve	-ve	+ve	+ve	+ve	A/GH_2S	+ve	nd
+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	nd
+ve	nd	nd	nd	nd	nd	nd	nd	nd	+ve
+ve	+ve	nd	nd	nd	nd	+ve	nd	nd	nd

MR: methyl red; VP: Voges-Prausker; TSI: triple sugar iron; NT: nitrate reduction; BE: bile esculin; A/G: acid and gas production; A/GH2S: acid-gas and hydrogen sulfide production; K/A: alkaline and acid production; nd: not done; +ve: positive; -ve: negative

Table.3 Qualitative phytochemical analysis of cold extracts of *B. monosperma* with different solvents

Constituents	**PE**	**AC**	**EOH**	**MeOH**	**H_2O**
Alkaloids	+ (+)	- (-)	+ (+)	+ (+)	+ (+)
Carbohydrates	+ (+)	- (-)	+ (+)	- (-)	+ (+)
Flavonoids	- (-)	- (-)	- (-)	+ (+)	- (-)
Glycosides	- (+)	- (-)	- (+)	- (-)	- (+)
Proteins	- (-)	- (-)	- (-)	- (-)	- (-)
Saponins	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
Starch	- (-)	+ (+)	- (-)	- (-)	+ (+)
Sterols	- (-)	- (-)	- (-)	- (-)	- (-)
Tannins	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)

PE: petroleum ether; AC: acetone; EOH: ethanol; MeOH: methanol. In parenthesis, status of phytoconstituent in hot extract is given. +: presence; -: absence of phytoconstituent.

Table.4 Antibiogram of clinically isolated 9 bacteria by the disc-diffusion method with two sets of antibiotics.

Bacterium	**First set of antibiotics**												
	G	Nx	Nf	Ak	Ce	I	Pt	Gf	Of	Nt	Ac	Va	Ox
Acinetobacter sp.	S	S	S	S	S	S	R	S	S	S	S	-	-
C. freundii	R	S	S	S	R	R	R	R	R	R	R	-	-
E. coli	R	R	S	S	R	R	R	S	R	S	R	-	-
K. pneumoniae	R	R	S	S	R	R	R	S	R	S	R	-	-
P. aeruginosa	R	R	R	S	R	S	S	S	R	R	R	-	-
MRSA	R	S	S	R	R	R	R	R	S	R	S	S	R
VRSA	R	S	S	S	R	R	R	R	S	R	R	R	R
S. aureus	R	S	S	S	R	R	R	R	S	R	S	S	S
	Second set of antibiotics												
Enterococcus sp.	Le	Ac	Ctr	Azm	L	Of	G	Ne	Ak	Nf	Nx	Of	-
	R	S	S	R	R	R	R	S	R	S	S	R	-

Antibiotics (µg/disc): Ac: amoxyclav 30; Ak: amikacin 30; Azm: azithromycin 15; Ctr: ceftriaxone; G: gentamicin 30; Gf: gatifloxacin 30; I: imipenem 10; L: lincomycin 10; Le: levofloxacin 5; Ne: neomycin 30; Nf: nitrofurantoin 300; Nt: netilmicin 30; Nx: norfloxacin 300; Of: ofloxacin 5; Ox: oxacillin 1; Pt: piperacillin/ tazobactam 100/10; Va: vancomycin 30. For *S. aureus*, oxacillin and vancomycin was used individually and lawns had no inhibition zone. R: resistance and S: sensitivity of a bacterium; -: antibiotic was not used. Data of the second repeated experiment are presented. All values are mean of duplicate readings.

Table.5 Size of inhibition zones of cold leaf-extracts with different organic solvents of B. monosperma against different bacteria (mm)

Bacteria	PE	AC	EOH	MeOH	H_2O	Ciprofloxacin 30 µg/disc
Acinetobacter sp.	12 (12)	14 (14)	16 (15)	16 (16)	14 (14)	18
C. freundii	12 (12)	13 (13)	14 (17)	15 (15)	14 (14)	16
E. coli	- (-)	- (-)	10 (11)	12 (12)	14 (14)	18
K. pneumoniae	13 (13)	13 (13)	16 (16)	15 (15)	16 (16)	17
P. aeruginosa	12 (13)	13 (13)	10 (11)	16 (16)	15 (15)	18
Enterococcus sp.	14 (15)	14 (14)	16 (16)	14 (14)	19 (21)	18
S. aureus	14 (15)	12 (12)	14 (14)	14 (14)	16 (16)	19
MRSA	13 (14)	12 (12)	13 (13)	14 (14)	15 (15)	18
VRSA	12 (14)	13 (13)	13 (12)	12 (12)	13 (13)	18

PE: petroleum ether; AC: acetone; EOH: ethanol; MeOH: methanol; in parenthesis, sizes of inhibition zones in hot extracts are given. -: absence of inhibition.

Table.6 MIC and MBC values of cold and hot leaf-extracts with different solvents against MDR bacteria (mg/mL)

Bacteria	PE		AC		EOH		MeOH		H_2O	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Acinetobacter sp.	2.62 (13.30)	5.91 (30.00)	5.91 (2.62)	13.30 (5.91)	2.62 (5.91)	5.91 (13.30)	2.62 (2.62)	5.91 (5.91)	2.62 (2.62)	5.91 (5.91)
Citrobacter sp.	5.91 (2.62)	13.30 (5.91)	0.52 (2.62)	1.16 (5.91)	5.91 (5.91)	13.30 (13.30)	2.62 (2.62)	5.91 (5.91)	5.91 (5.91)	13.30 (13.30)
E.coli	ND (ND)	ND (ND)	ND (ND)	ND (ND)	2.62 (13.30)	5.91 (30.00)	5.91 (5.91)	13.30 (13.30)	5.91 (5.91)	13.30 (13.30)
K.pneumoniae	13.30 (5.91)	30.00 (13.30)	2.62 (5.91)	5.91 (13.30)	5.91 (5.91)	13.30 (13.30)	1.16 (1.16)	2.62 (2.62)	2.62 (2.62)	5.91 (5.91)
P.aeruginosa	0.52 (5.91)	1.16 (13.30)	2.62 (5.91)	5.91 (13.30)	1.16 (2.62)	2.62 (5.91)	1.16 (1.16)	2.62 (2.62)	5.91 (5.91)	13.30 (13.30)
Enterococcus sp.	1.16 (2.62)	2.62 (5.91)	1.16 (2.62)	2.62 (5.91)	5.91 (5.91)	13.30 (13.30)	5.91 (5.91)	13.30 (13.30)	0.52 (0.23)	1.16 (0.52)
S.aureus	2.62 (5.91)	5.91 (13.30)	2.62 (0.52)	5.91 (1.16)	5.91 (2.62)	13.30 (5.91)	2.62 (2.62)	5.91 (5.91)	5.91 (5.91)	13.30 (13.30)
MRSA	2.62 (2.62)	5.91 (5.91)	2.62 (2.62)	5.91 (5.91)	5.91 (1.16)	13.30 (2.62)	5.91 (5.91)	13.30 (13.30)	5.91 (5.91)	13.30 (13.30)
VRSA	2.62 (13.30)	5.91 (30.00)	2.62 (1.16)	5.91 (2.62)	1.16 (5.91)	2.62 (13.30)	5.91 (5.91)	13.30 (13.30)	5.91 (5.91)	13.30 (13.30)

Spontaneous mutation in bacteria occurs at the rate, 1 in 10^7 cells usually. Eventually, some drug-resistant mutant predominates with the replacement of all sensitive strains by the resistant strain, the later serving as if a doppelgänger. Since, the emergence of resistant mutants is a self-repetitive process in conditions ideal for pathogens, serial/continual resistant events to a gamut of diverse antibiotics land at the emergence

of multidrug resistance in a bacterium, at least in an aged/immune-compromised body. Indeed, the horizontal transfer of genetic materials from one organism to another appears faster than mutational changes, a phenomenon popularly called as, evolution of quantum leaps, operates naturally (Groisman EA, 1996). It is because, genes for the drug-resistance mechanism are operative in antibiotic-producing cells, and those are transferred naturally to sensitive strains (Martinez JL, *et al* ., 2002), as an event of natural selection. Ultimately, antibiotic resistance remains as the clinical determinant of the pathogenesis. Slowly, the use of numbers of antibiotics for the control of infectious diseases in last decades have led to multiple resistances in one cell, the MDR strain of a species, paradigmatically with any of notorious pathogens. As conjectured from retrospective follow-ups, it is clear that older antibiotics slowly became obsolete, by the resistant mechanism. The clinical concern is that antibiotic resistance was reported in several pathogenic bacteria for which, particular antibiotics were never applied. Is this the mechanism of the transformation of a harmless commensal to a perilous MDR pathogen in the present antibiotic era? Not surprisingly, drug resistant bacteria gain the capability of surviving and multiplying under stress conditions. The biological rule, any limiting condition for the majority would be an excellent opportunity for the minority. When in presence of a drug *in vivo*, all the drug sensitive strains are eliminated and the resistant strain survives, multiplies, and predominates, culminating in a disease. Drug resistant strains and their control by newer antibiotics are leitmotivs in the odyssey of the emergence of MDR and PDR strains of umpteen pathogens in the last 4-5 decades and more. MDR-MRSA is the intractable, ghoulish example rising to a great notoriety of being marked as the superbug of health domain, worldwide (Dubey D, *et al*., 2013).

In conclusion from the recorded data, it could be taken that *B. monosperma* leaf-extract could be used in treating infectious diseases, caused by the range of tested bacteria, as complementary and alternate medicine, since crude phyto-extracts of the plant could not be breached by MDR pathogenic bacteria. Apothecary would benefit from these findings of the plant for drugs of finesse, *i.e.*, non-microbial antimicrobials in the crusade against MDR pathogens.

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