Antibacterial and Phytochemical Screening of *Piper cubeba* Linn. f. (Kababchini)

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Abstract

Present study provides an in-vitro efficacy of *Piper cubeba* Linn. f. (Kababchini) in terms of its antibacterial activity against various gram positive and gram negative bacterial strains using Agar well and Kirby Bauer's Disk Diffusion method according to CLSI guidelines. The efficacy was compared with the standard drugs used as positive control viz. Ciprofloxacin for gram positive strains and Gentamicin as gram negative strains and the solvent used to dissolve the test drug-Dimethyl Sulphoxide (DMSO) as Negative Control. Phytochemical analysis was also done to confirm the presence of various phytoactive constituents present in the test drug and it was revealed that Kababchini contains alkaloids, glycosides, carbohydrates, proteins, tannins, resins and terpenes. It was found to be sensitive towards *Staphylococcus aureus, S. epidermidis, Streptococcus mutans, Escherichia coli, Klebseilla pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa*. The study demonstrates the in-vitro efficacy of Kababchini to be considered as effective drug target for further screening and its wider use in Unani medicine to combat many infectious diseases effectively.

Keywords: Phytochemical, Antibacterial screening, *Piper cubeba* Linn. f. (Kababchini)

Introduction

Antimicrobial resistance is a serious problem in terms of clinical and public health. In-spite the successes of the Millennium Development Goals since 2000, the inhabitants of low-income countries still suffer an enormous burden of disease owing to diarrhea, pneumonia, HIV/AIDS, tuberculosis, malaria and other pathogens (Fig.1). Regardless of the origin of resistance, the fact is that the number of new resistant microorganisms grows faster than the development of new drugs. Therefore, the search for alternative therapies is imperative. In this direction Unani system of medicine (USM) claims to possess a number of effective and safe drugs useful in the treatment of infectious diseases. Unani physicians are using these drugs successfully for centuries for the treatment of infectious diseases with significant recovery and without any side effects. However, there is a need to provide all such drugs scientific basis through systemic investigations using modern tools and experimental techniques. Some of the medicinal plants investigated for their antimicrobial effect, particularly those used in (USM) as blood purifier (Musaffiye-dam) includes Chirayita [*Swertia chirayita* Linn. (Gentianaceae)], Banafshah (*Viola odorata* Linn. (Violaceae)], Shahtra [*Fumaria officinalis* Linn. (Fumariaceae)], Bakayn [*Melia azadirachta (Meliaceae)*], Sambhalu

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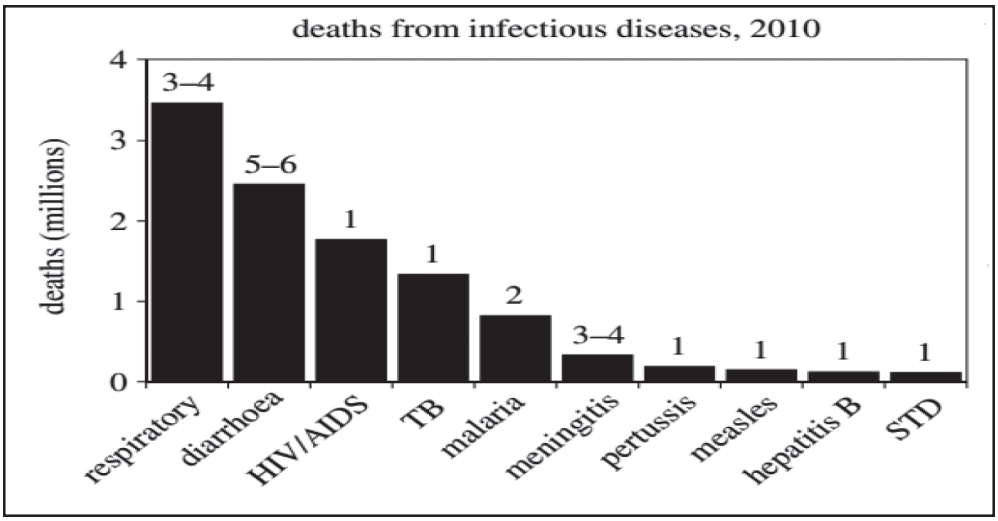

Figure 1: Top 10 causes of death from infectious diseases in 2010
Fig. above bars are numbers of pathogens causing majority of deaths from each disease
(Data courtesy: Dye C, 2014)

[*Vitex negundu* Linn. *(Verbenaceae)*], Khatmi [*Althaea officinalis* Linn. *(Malvaceae)*], Black pepper [*Piper nigrum* L. (Piperaceae)]; Clove [*Syzygium aromaticum* (L.) Merr.& Perry (Myrtaceae)], Nutmeg [*Myristica fragrance* Houtt. (Myrtaceae)], Oregano [*Origanum vulgare* ssp. *hirtum* (Linn.) Letsw. (Lamiaceae); Thyme [*Thymus vulgaris* L. (Lamiaceae)] and others (Dorman and Deans, 2000; Latif, 2010). However, there appears little work done on *Piper cubeba* Linn. f. fruits to evaluate its antimicrobial properties (Khan and Siddiqui, 2007). Hence, the present studies were carried out to re-assess the antimicrobial efficacy of *Piper cubeba* Linn. f. focusing to compare its effect upon bacterial growth with the known antibiotics i.e. Ciprofloxacin (for gram positive bacterial strains) and Gentamicin (for gram negative bacterial strains). Furthermore, the study also re-confirms the reported literature claims of therapeutic efficacy and presence of different phyto-constituents having beneficial effect.

Review of literature

Botany: *Piper cubeba* Linn. f. (Family-Piperaceae) originally introduced to Europe as a spice; cultivated to a small extent in Assam and Karnataka states of India (Anonymous, y.n.m). Commercial cubebs consist of the dried berries are similar in appearance to black pepper, but with stalks attached - the “tails” in “tailed pepper”. The dried pericarp is wrinkled; fruit or berries are nearly globular, rough, grayish somewhat lighter-colored than black pepper, of a rather pleasant, aromatic odor and a hot bitter and somewhat camphoreous taste.

Ethno-pharmacological reports: Literature on ethno-pharmacological uses of Kababchini mentions that the drug has been therapeutically used in acute rhinitis,

catarrh, cough, chronic bronchitis, asthma, cystitis, diarrhea, dysentery, chronic headache, hay fever, helminthiasis, digestive problems, dysmenorrhoea, diseases of liver and spleen etc. Further the ethno-medicinal literature also mentions cubebs as antiseptic, antimicrobial and anti-infective or as blood purifier used in many infectious diseases of the body (Khan, 1313H; Ibne Baitar, 1197; Husain 1285 H; Chopra, 1956; Dey, 1988, Kantoori, 1992; Rastogi and Mehrotra, 2002; Evans, 2002; Anonymous, 2005).

Use in traditional medicine: The literature review undertaken on the test drug Kababchini reveals that the drug has been traditionally used in many infectious diseases as catarrh, rhinitis, bronchitis, hay fever, cystitis, stomatitis, urethritis (Anonymous, 2005; Nadkarni, 1982; Chopra, 1956; Ahmad *et al.*, 2012). Therefore, in-vitro study was done to evaluate its antimicrobial potential in Microbiology Lab., Department of Ilmul Advia, A.M.U., Aligarh during 2014, aimed to re-validate use of this well-known Unani drug against infectious diseases.

Material and Methods

Plant Material: Dried berries of *P.cubeba* were procured from the local market of Baradari in Aligarh city and were botanically identified (Fig.2). Berries were washed with DDW and dried at room temperature in a ventilated room, milled to coarse powder and stored in a close air tight container in dark until use.

Figure 2: Kababchini (Piper cubeba Linn.)

Preparation of Plant extract: The coarse drug material was extracted with 95% ethanol as a solvent at 50^0C for 6 hours and dried under reduced pressure in the Lypholizer. Extraction was done according to the method described by Afaq *et al., (*2000) and Peach and Tracey (1955) with some minor modifications, keeping in mind that the thermo labile elements present in the drugs are destroyed when exposed to a higher temperature beyond 55^0C, so the heat wherever was needed was kept as low as possible to prevent the loss of thermo-labile substances present in the drugs from destruction. Stock solution was prepared from the dried extract so obtained in the Dimethyl Sulphoxide (DMSO) as a solvent for use. The respective stock solutions so prepared were refrigerated till further use. Strict aseptic precautions were followed throughout the process.

Phytochemical analysis: Phytochemistry also helps in standardizing the herbal preparations so as to get the optimal concentrations of known active constituents, and in preserving their activities (Rehman and Latif, 2015). Qualitative analysis of the chemical constituents present in the drug sample (Bhattacharjee and Das, 2005 and Afaq*et al*., 1994) Standard tests and methodology has been followed to confirm the presence of chemical constituents (table-1).

Antibacterial Susceptibility Testing: Antimicrobial susceptibility testing was done by Kirby Bauer's disk diffusion method (1996) as was performed on biofilm isolates by Kirby-Bauer on Muller Hinton Agar (Barry *et al*., 1999). It is a highly standardized test approved by the Food and Drug administration for determining the susceptibility of bacteria to an antimicrobial agent.

Bacterial strains used for the study are listed in table-2&3. The standard medium Mueller Hinton Agar, was poured to a depth of 4 mm in a 90 mm petridish. The plate was inoculated by streaking the entire surface in three planes with a sterile cotton swab (PW041, Himedia Labs Pvt. Ltd., Mumbai, India) dipped into standardized inoculums, spreaded evenly with the help of L-Spreader (PW1085, Himedia Labs Pvt. Ltd., Mumbai, India). The bacterial inoculum was prepared from an 18 hour broth culture of the microbe to be tested and was standardized with sterile physiologic saline to contain 10^6 cfu/ml. Standardized commercial paper disk containing amounts of the antimicrobial agents to be tested were placed on the surface of the agar. The plate was incubated in an inverted position at 37^0C for 18 hours. The diameter of zone of inhibition produced by the drug was measured with the help of Antibiotic zone scale (PW297, Himedia Labs Pvt. Ltd., Mumbai, India) from each disk (Kingsbury and Wagner, 1990).

Statistical Analysis: All the statistical analysis was done using gpaid software, One way ANOVA and the post test named Bonferroni: Selected pairs of column with multiple comparison was performed with p-value <0.05.

Results and Discussion

Unani System of Medicine (USM) has a holistic approach of treatment, utilizes drugs of natural origin used as whole either as powder, decoction or infusion in spite of isolating phyto-constituents of interest which is a common practice in Western medicine. In this provision of USM, the phytochemicals which are believed to play a major role in disease protection appear to work alone and in combination, and perhaps in conjunction, to prevent, halt, or lessen disease. This makes it important to analyze them in drugs of common use. Therefore, phytochemical analysis is necessary for standardization and this also helps in understanding the significance of phyto-constituents in terms of their medical efficacy observed activities.

Phytochemical analysis of active constituents present in the drug revealed that it contained alkaloids, phenols, resins, saponins, sugars and tannins (Table-1). These were the encouraging results for our study as such types of chemical compounds are usually responsible for the antimicrobial activity. As can be realized from the fact that alkaloids possess antimicrobial and anti-inflammatory activity; flavonoids

Table 1: Qualitative Analysis of the Phytochemicals

S.No.	Chemical Constituents	Test Reagents	Inference
			Kababchini (*P.cubeba* Linn.)
1.	Alkaloids	Dragendorff's reagent	+
		Wagner's reagent	+
		Mayer's reagent	+
2.	Carbohydrates	Molish Test	+
		Fehling Test	+
		Benedict Test	+
3.	Flavonoids	Mg Ribbon and dil. Hcl	_
4.	Glycosides	NaOH Test	+
5.	Tannins/Phenols	Ferric Chloride Test	_
		Liebermann's test	+
		Lead Acetate test	+
6.	Proteins	Xanthoproteic test	_
		Biuret test	+
7.	Starch	Iodine Test	_
8.	Saponins	Frothing with $NaHCO_3$	_
9.	Steroids/Terpenes	Salkowski Reaction	+
10.	Resins	Acetic anhydride test	+

Indications: '–'Absence and '+' Presence of constituents

act as an anti-oxidant and antimicrobial, protect plants from UV radiations and micro-organisms (Cushnie and Lamb, 2005); tannins precipitate proteins, prevents the development of bacteria and since proteins are necessary for their growth and development so, they not only stop their nutrition but also precipitate their own proteins (Said, 1996) and saponins-the soapy substances are general cleansers, have antiseptic properties (Hirat and Suga, 1983). Most of these findings were found to be helpful in understanding and showing its biological activity. Various other studies have also been done in past with other isolated phyto-chemicals as lignoids, cubebin and other metabolites present in the drug studied, which reported significant pharmacological activity (Parmar *et al*., 1997; Arruda *et al*., 2005).

Present study is a step ahead in this direction to scientifically validate the antimicrobial activity of Kababchini, using Agar well and Kirby Bauer's Disk diffusion methods against different Gram Positive and Gram Negative bacterial strains. A total volume of 40 µl of test drug from concentrations viz. 40.0 µg/ml was used and compared with the standard drug Ciprofloxacin (30µg) for Gram Positive and Gentamicin (30 µg) for Gram Negative bacteria and Plane control i.e. DMSO-(Dimethyl Sulphoxide) the solvent used (to exclude out any activity due to the solvent used to dissolve the plant extract). Ethanolic extract of Kababchini exhibited a prominent significant inhibitory activity against most of the strains and produced large inhibitory zone. Gram positive strains *S.epidermidis* (13.8±1.31) >*S.aureus* (11.8±1.02) > *S.mutans* (9.4±0.67), while it was completely resistant to *S.pyrogenes*, *B.cereus*, *C.xerosis* (Table-2; Fig 3). For Gram negative strain used,

Table 2: Antibacterial Activity of Ethanolic Extracts of Kababchini (*Piper cubeba*) against Gram positive bacterial strains

S. No.	Test strains	Zone of Inhibition (in mm) expressed as Mean ± S.E.M (S.D) Probability of error		
		Drug Extract (µg/ml)	Control (DMSO- 50µl)	Standard (Ciprofloxacin 30µg)
1.	*S.mutans* (*ATCC* 25175)	9.4±0.67(1.51)* (S)	6.6±0.24(0.54) (R)	21.2±0.37(0.83) (S)
2.	*S.epidermidis* (ATCC 155)	13.8±1.31(2.95)* (S)	6.4±0.24(0.54) (R)	21.6±0.24(0.54) (S)
3.	*S.pyrogenes* (ATCC 14289)	6.4±0.24(0.54) (R)	6.4±0.24(0.54) (R)	21.2±0.37(0.83) (S)
4.	*B.cereus* (ATCC 11778)	6.6±0.24(0.54) (R)	6.6±0.24(0.54) (R)	21.4±0.24(0.54) (S)
5.	*S.aureus* (ATCC 29213)	11.8±1.02(2.28)*** (S)	6.6±0.24(0.54) (R)	26.8±0.20(0.44) (S)
6.	*C.xerosis* (ATCC 373)	6.6±0.24(0.54) (R)	6.6±0.24(0.54) (R)	21.2±0.37(0.83) (S)

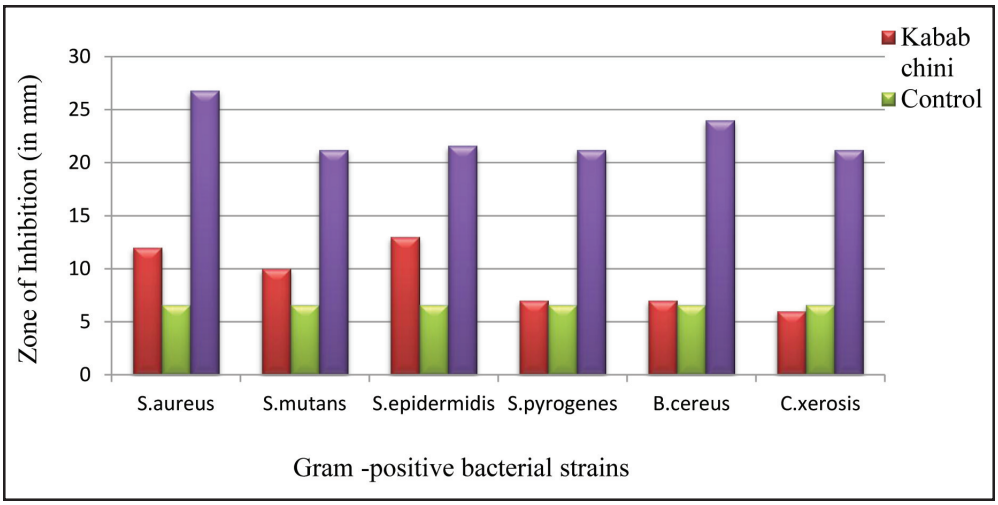

Figure 3: Antibacterial activity of ethanolic extracts of the test drugs against Gram positive strains

it showed sensitivity to all strains and there was equal inhibitory activity in the order of *P.aeruginosa* (9.6±0.24) >*P.vulgaris* (9.4±0.67) >*K.pneuomoniae* (9.4±0.67) >*E.coli* (8.2±0.48). All showed a significant inhibition as compared to Gentamicin (ZOI- 14.0-14.8 mm) (Table-3; Fig 4). In-vitro screening and phytochemicals' presence also attributes and confirms medicinal efficacy as uses reported earlier (Gayatri and Sahu, 2011; Tamadher, 2013; Rezende *et al.*, 2016) who had tested various extracts and at 400µg/disk, the ZOI was 19 mm against *S.aureus* for dichloromethane fraction, so it indicates that these tested drugs have great potentiality and can be used in skin and dental infections, too.

Table 3: Antibacterial activity of ethanolic extracts of Kababchini (*Piper cubeba*) against Gram negative bacterial strains

S. No.	Test strains	Zone of Inhibition (in mm) expressed as Mean ± S.E.M (S.D)Probability of error		
		Drug Extract (µg/ml)	Control (DMSO- 50µl)	Standard (Gentamicin 30µg)
1.	*E.coli* (ATCC 25922)	8.2±0.48(1.09)*** (S)	6.4±0.24(0.54) (R)	14.8±0.20(0.44) (S)
2.	*P.vulgaris* (ATCC 6380)	9.4±0.67(1.51)* (S)	6.4±0.24(0.54) (R)	14.0±0.54(1.22) (S)
3.	*P.aeruginosa* (ATCC 25619)	9.6±0.24(0.54)* (S)	6.4±0.24(0.54) (R)	14.8±0.20(0.44) (S)
4.	*K.pneuomoniae* (ATCC 15380)	9.4±0.67(1.51)* (S)	6.4±0.24(0.54) (R)	14.8±0.20(0.44) (S)

S = Sensitive R = Resistant

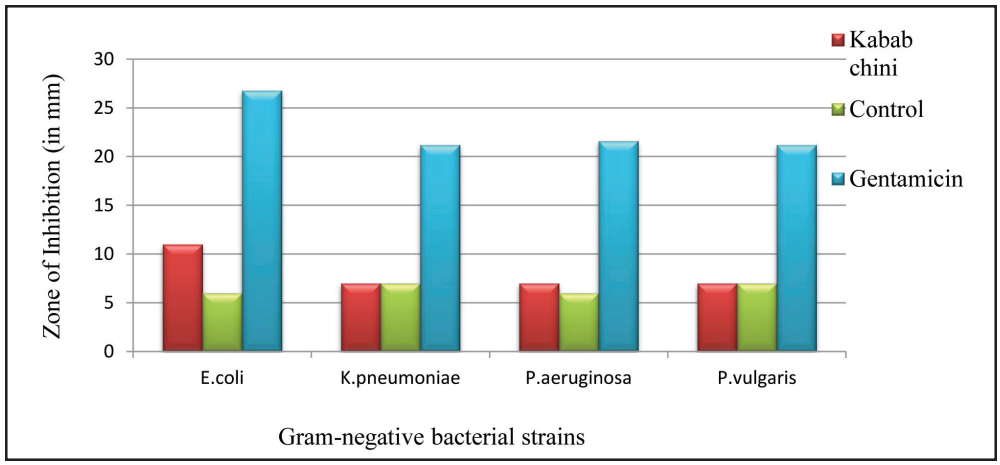

Figure 4: Antibacterial activity of ethanolic extracts of the test drugs against gram negative strains

Conclusion

An antibiotic assay done in the study reveals that 'Kababchini' has a significant antibitotic activity against S.*aureus, S.mutans, S.epidermidis, S.pyrogenes*and *B.cereus* while it showed a complete resistance to *C.xerosis*. Most significant sensitivity was shown towards *B.cereus*whileamong the gram negative strains it showed a sensitivity pattern to all bacterial strains used for the study viz. *E.coli, K.pneuomoniae, P.aeruginosa* and *P.vulgaris.* However, this inference is just a step ahead regarding scientific validation of 'Kababchini' and many such other studies are needed in this direction before its medical use to combat infectious diseases caused by such bacterial species.

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