Original Article

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Antimicrobial efficacy of fruit extracts of two *Piper* species against selected bacterial and oral fungal pathogens

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Abstract

Aim: To assess the antimicrobial efficacy of five solvent extracts of two *Piper* species commonly used in diet and traditional medicine, *P. cubeba* and *P. longum*, against selected bacterial and oral fungal pathogens i.e. *Streptococcus mutans, Staphylococcus aureus, Candida albicans* and *Saccharomyces cerevisiae*. **Methods:** The antimicrobial activity of five extracts of cubeb berries and Indian long pepper fruits was determined by the agar well diffusion method. The minimum inhibitory concentration (MIC) for the acetonic, methanolic and ethanolic extracts was determined by the modified agar well diffusion method. **Results:** Of the 5 fruit extracts evaluated, acetone, ethanol and methanol extracts of both the *Piper* spp. were found to have variable antimicrobial activities against all the four oral pathogens. The acetonic fruit extract of *P. cubeba* was the most effective against both the yeasts with the highest zone of inhibition (15.31 mm) against *C. albicans* followed by the methanolic (12.31 mm) and ethanolic (11.94 mm) extracts. *C. albicans* was found to be most sensitive pathogen, which survived up to 6.25 mg/mL in the acetonic extract (MIC = 12.5 mg/mL) followed by the methanolic and ethanolic extracts (MIC = 25 mg/mL). The acetonic, methanolic and ethanolic extracts of *P. longum* fruits showed almost equal inhibition zones of both yeasts, ranging between 10.64 and 14 mm. *C. albicans* survived up to 12.5 mg/mL (MIC= 25 mg/mL) while *S.cerevisiae* survived up to 25 mg/mL (MIC = 50 mg/mL). **Conclusions:** The crude extracts obtained from the fruits of the two *Piper* spp. may be used to treat oral fungal species, especially *C. albicans*, as they produced larger inhibition zones than antifungal drugs often used to treat these pathogens.

Keywords: oral pathogens, *Piper cubeba, Piper longum*, antibacterial or antifungal activity, minimum inhibitory concentration (MIC).

Introduction

Oral diseases continue to be a major health problem worldwide[1]. Dental caries and periodontal diseases are among the most important global oral health problems[2]. *Streptococcus mutans* is the major organism implicated with dental caries and oral infections[3]. *Candida albicans* is the most common yeast isolated from the oral cavity, and is associated with oral fungal infections, endocarditis and septicemia[4]. *Staphylococcus aureus,* a major human pathogen, is responsible for a number of hospital-acquired infections and propagates mainly in mouth and hands in the hospital environment[5-6]. *Saccharomyces cerevisiae* considered to be an opportunistic pathogen in the oral cavity, may induce significant oral

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risks by acting as a tertiary colonizer in the progress of dental caries thus causing both superficial and invasive infections[7].

Microbial resistance to most of the antibiotics commonly used to treat oral infections (penicillins and cephalosporins, erythromycin, tetracycline and derivatives and metronidazole) has been documented[8]. The resistance of microorganisms against the traditional antibiotics needs urgent attention for the development of the new drug molecules. It is well documented from ancient times that active principles from plant origin have been used as medicines for various diseases and microbial infections[9]. A wide variety of medicinal plants used traditionally have not yet been systematically investigated against various microbial pathogens[10].

The genus *Piper* of family *Piperaceae*, with over 1,000 species, is distributed in both hemispheres. *Piper cubeba* Linn., commonly known as cubeb, tailed pepper (due to the stalks attached), Java pepper (in Java) and kemukus (in Indonesia), is a climbing perennial plant[11]. The fruits of this plant are used as a spice and have medicinal value, being often used for the treatment of abdominal pain, asthma, chronic bronchitis, diarrhea, dysentery, gonorrhea, enteritis and syphilis and reported to have an inhibitory effect on hepatitis C virus protease[12-13]. The dried fruits contain up to 10% essential oil composed of monoterpenes (sabinene 50%, carene, α-thujene, 1,4-cineol and 1,8-cineol) and sesquiterpenes (copaene, α- and β-cubebene, δ-cadinene, caryophyllene, germacrene, cubebol)[14].

P. longum Linn. (Javanese, Indian long pepper, pippali), another medicinal plant belonging to the genus *Piper,* is a small shrub characterized by fruits called berries borne in fleshy spikes, oblong, blunt and blackish green in color[15]. The fruits contain 1% volatile oil, resin, a waxy alkaloid, a terpenoid substance and alkaloids piperine and piperlongumine[16]. It is widely used as a folk medicine to cure diseases such as leprosy, tuberculosis, gonorrhea, paralysis of the tongue, diarrhea, cold, palsy, gout, rheumatism, lumbago, insomnia, epilepsy, anorexia, piles, dyspepsia, leucoderma cholera, scarlatina, chronic malaria, viral hepatitis, bronchitis, cough, asthma, stomachache, spleen diseases and tumors[17-18].

A literature search reveals that *Piper* spp. have been used in traditional medicine since long for several ailments[15,1820] however, not much work has been done on the antimicrobial activity of their fruits. Since *P. cubeba* and *P. longum* have been used traditionally in medicine and their fruits have been used as food material, the biological evaluation of the fruits of these plants may lead to development of safer therapeutic agents[21]. Therefore, the present study has been designed to assess the antimicrobial efficacy of fruit extracts of *P. cubeba* and *P. longum* against selected bacterial and oral fungal pathogens.

Material and methods

Fruits/catkin of *P. cubeba* and *P. longum* were collected from the local market of Delhi, India. Dr. B.D. Vashishta (Botany Department) Kurukshetra University, Kurukshetra confirmed the identification of the specimens.

Extraction

The samples were carefully washed under running tap water followed by sterile distilled water, and were air dried at room temperature (40°C) for 5 days and pulverized to a fine powder using a sterilized mixer grinder and stored in air-tight bottles. Four different solvents, namely ethanol, methanol, acetone and aqueous solvent (hot and cold), often used for the extraction of plant material were used to obtain the extracts[22]. An amount of 10 g of pulverized fruit was separately soaked in 100 mL of acetone, ethanol, methanol, and cold sterile distilled water for 24 h. The same amount of pulverized fruit (10 g) was immersed in 100 mL of hot sterile distilled water (100°C) and allowed to stand for 30 min in a water bath with occasional shaking, and then left undisturbed for 24 h. Each preparation was filtered through a sterilized Whatman No.1 filter paper, and the filtered extract was concentrated under vacuum below 40°C using Heidolph, VE-11 rotaevaporator[23-25]. The obtained dried extract was exposed to UV rays for 24 h and checked for sterility on nutrient agar plates and stored in labeled sterile bottles in a freezer at 4°C until further use[26].

Test microorganisms

Two oral pathogenic bacteria - *S. mutans* (MTCC*497) and *S. aureus* (MTCC 740) - and two oral pathogenic yeasts - *C. albicans* (MTCC 227) and *S. cerevisiae* (MTCC 170) - were obtained from Microbial Type Culture Collection, IMTECH, Chandigarh. The microorganisms were subcultured on the specific media recommended for different microorganisms such as Brain heart infusion agar (*S. mutans*), Nutrient agar (*S. aureus*), Malt yeast agar (*C. albicans* and *S. cerevisiae*) and incubated aerobically at 37°C. The media were procured from HiMedia Laboratory Pvt. Ltd., Bombay, India. Identification of all the strains was confirmed by standard biochemical and staining methods[27-29].

Screening for antimicrobial activity

Antimicrobial activity of the 5 extracts of cubeb berries and Indian long pepper fruits was determined by following the agar well diffusion method of Okeke[30]. In this method, pure isolate of each microbe was subcultured on the recommended specific medium for each microorganism at 37°C for 24 h. A plate of each microorganism was taken and a minimum of 4 colonies were touched with a sterile loop and transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted equal to that of 10^6cfu/mL (standardized by 0.5 McFarland standard) and used as the inoculum for performing agar well diffusion assay. One hundred microliters of inoculum of each test organism was spread onto the specific media plates so as to achieve a confluent growth. The agar plates were allowed to dry and 8-mm-diamater wells were made with a sterile borer in the inoculated agar plates. The lower portion of each well was sealed with a little specific molten agar medium[31]. The dried fruit extracts were reconstituted in 20% dimethylsulfoxide (DMSO) for the bioassay analysis[30].

A 100 µL volume of each extract was propelled directly into the wells (in triplicates) of the inoculated specific media agar plates for each test organism. The plates were allowed to stand for 10 min for diffusion of the extract to take place and incubated at 37°C for 24h[32-34]. Sterile DMSO served as the negative control, and ciprofloxacin (for bacteria) and amphotericin-B (for fungi) served as the positive controls. The antimicrobial activity, indicated by the formation of an inhibition zone surrounding the well containing the extract, was recorded if the inhibition zone was greater than 8 mm. The experiments were performed in triplicates and the mean values of the diameter of inhibition zones with ± standard deviation were calculated[35-36].

Determination of minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration of a compound/extract/drug that completely inhibits the growth of the microorganism in 24 h[35]. The MIC for the acetonic, methanolic and ethanolic fruit extracts was determined by following the modified agar well diffusion method[29]. A twofold serial dilution of each extract was prepared by first reconstituting the fruit extract in 20% DMSO followed by dilution in sterile distilled water to achieve a decreasing concentration range of 50 mg/mL to 0.39 mg/mL. A 100 µL volume of each dilution was introduced into wells (in triplicate) in the specific media agar plates already seeded with 100 µL of standardized inoculum (10^6cfu/mL) of the test microbial strain. All test plates were incubated aerobically at 37°C for 24 h and observed for the inhibition zones. The lowest concentration of each extract showing a clear zone of inhibition (>8 mm) (in triplicate), considered as the MIC, was recorded for each test organism[33].

Statistical analysis

The results are presented as mean ± standard deviation. One-way ANOVA followed by Dennett's *t*-test for multiple comparisons were used for statistical evaluation. P values less than 0.05 were considered significant.

Results and discussion

The results of antimicrobial properties of ethanol, methanol, acetone and aqueous (hot and cold) fruit extracts of *P. cubeba* and *P. longum*, the positive control ciprofloxacin (for bacteria) and amphotericin-B (for fungi), and the negative control (DMSO), are presented in Table 1 and values of MIC of these extracts against the test pathogens are presented in Table 2. The antimicrobial activity of *P .cubeba* and *P. longum* extracts on the agar plates varied for the different solvents. Both positive controls produced significantly larger inhibition zones against the test bacteria (ciprofloxacin) and yeasts (amphotericin-B). However, the negative control produced no observable inhibitory effect. Of the 10 fruit extracts of *P. cubeba* and *P. longum* screened for antibacterial and antifungal activity, acetone, methanol and ethanol extracts showed activity against both the bacteria (*S. mutans* and *S. aureus*) and both yeasts (*C. albicans* and *S. cerevisiae*). However, aqueous extracts, both hot and cold, showed no activity against the test strains (Table 1).

A perusal of the data (Table 1) reveals that the acetonic fruit extract of *P. cubeba* was the most effective against both yeasts. It showed the largest zone of inhibition (15.31 mm) against *C. albicans* (Figure 1a) followed by the methanolic (12.31 mm) and ethanolic extract (11.94 mm). *C. albicans* was found to be most sensitive pathogen, which survived up to 6.25 mg/mL in the acetonic extract (Figure 2 i) (MIC = 12.5 mg/mL) followed by the methanolic and ethanolic extracts (25 mg/mL). The inhibition zones produced by the 3 solvents against *S. cerevisiae* ranged between 14 and 12.94 mm. *S. cerevisiae* was found to be comparatively more resistant than *C. albicans*, as it survived up to 12.5 mg/mL (MIC = 25 mg/mL) in all 3 extracts tested. Interestingly, the acetonic fruit extract of *P. cubeba* showed comparatively greater activity against both yeasts, *C. albicans* (15.31 mm) and *S. cerevisiae* (14 mm), than that of the standard drug amphotericin-B (13 mm), which indicates a great potential against oral fungal pathogens. Among the tested fruit extracts of *P. cubeba*, the acetonic extract showed greater antibacterial

Table 1. Antimicrobial activity of *P. cubeba* and *P. longum* fruit extracts against oral pathogens determined by the agar well diffusion method on specific media for each test microorganism.

Solvent extracts (mg/mL)	Diameter of zone inhibition (mm)							
	S.mutans		*S.aureus*		*C.albicans*		*S.cerevisiae*	
	P.c.	*P.l.*	*P.c.*	*P.l.*	*P.c.*	*P.l.*	*P.c.*	*P.l.*
Acetone	12.64*±0.57†	10±0	18.96±1	10.64±0.57	15.31±0.57	11.31±0.57	14±0	10.93±1
Methanol	12.31±0.57	10±0	17.65±0.57	10±0	12.31±0.57	10.64±0.57	12.94±1	11.31±0.57
Ethanol	13±0	10.31±0.57	17.32±0.57	11.31±0.57	11.94±1	11.64±0.57	13.95±1	11.64±0.57
H A	-	-	-	-	-	-	-	-
C A	-	-	-	-	-	-	-	-
Ciprofloxacin	27.32±0.57	27.32±0.57	34.66±0.57	34.66±0.57	Nt	Nt	Nt	Nt
Amphotericin-B	Nt	Nt	Nt	Nt	13±0	13±0	11.94±1	11.94±1
20%DMSO	-	-	-	-	-	-	-	-

(-) = no activity, nt = not tested, *P.c.* = *Piper cubeba, P.l.* = *Piper longum,* HA= Hot aqueous extract, CA= Cold aqueous extract, Data shown as *Values, including diameter of the well (8 mm), are means of three replicates ± †Standard deviation, P = 0.001 indicates significantly different from control; Dennett's *t*-test after analysis of variance.

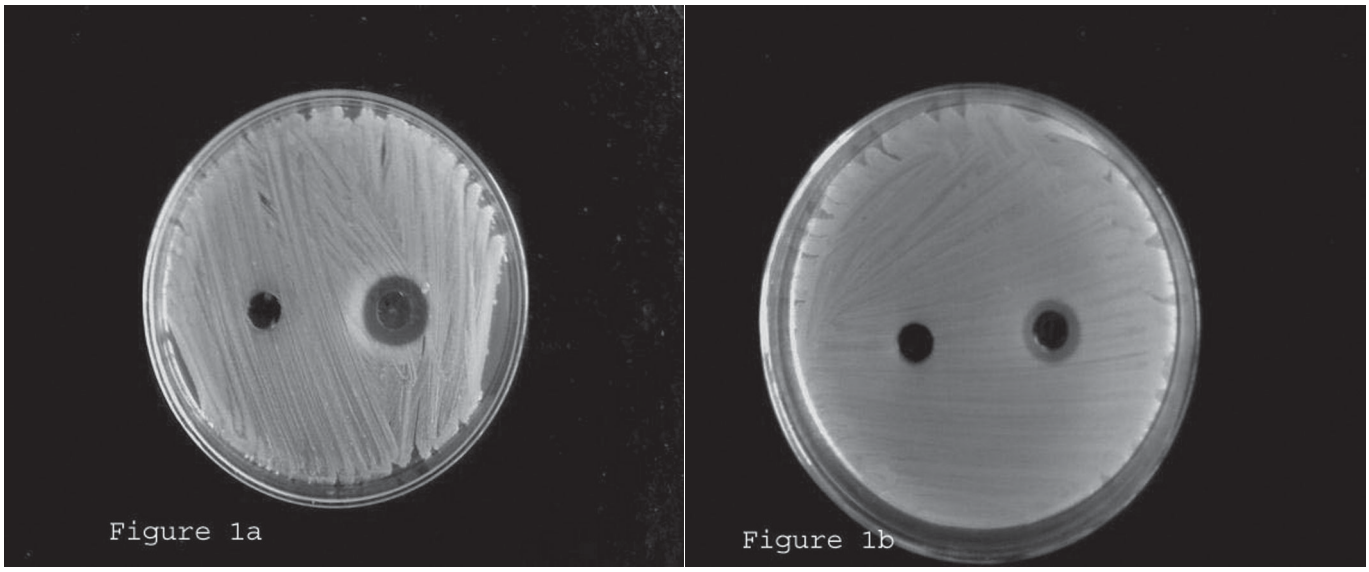

Fig. 1. Zone of antifungal inhibition against *C. albicans* shown by acetonic extract of *P. cubeba* (a) and ethanolic extract of *P. longum* (b), determined by agar well diffusion method and the control.

activity against *S. aureus,* the highest inhibition zone being 18.96 mm followed by the methanolic (17.65 mm) and ethanolic (17.32 mm) extracts (Table 1). *S. aureus* survived up to 12.5 mg/mL, thus having a MIC of 25 mg/mL (Table 2, Figure 2 ii). The inhibition zones produced by the 3 solvents against *S. mutans* ranged from 13 to 12.64 mm, and this microorganism survived up to 25 mg/mL (MIC = 50 mg/mL).

The aqueous (hot and cold) extracts did not show any activity against the tested yeast and bacteria (Table 1). A literature search reveals that among the *Piper* spp., the fruits of *P. cubeba* have received less attention on their antimicrobial activity. The berries contain essential oil consisting of monoterpenes, sesquiterpenes, the oxides 1.4- and 1, 8-cineole, kadsurin A, piperenone and the alcohol cubebol[37-38] in addition to two groups of secondary metabolites, i.e., alkaloids (piperine being the most abundant alkaloid[39]), and lignans (cubebin, though lesser in amounts in berries as compared to other lignans like yatein and hinokinin)[11,39-40]. The antibacterial and antifungal activity shown by the fruit extracts of *P. cubeba* against all the 4 test strains in this study may be due to the presence of piperine and cubebin in the berries. Cubebin has also been found to possess antiinflammatory, analgesic and trypanocidal activities[14, 41-42].

The data presented in the Table 1 reveal that acetonic, methanolic and ethanolic extracts of *P. longum* produced almost equal inhibition of both the yeasts, ranging between 10.64 and 14 mm (Figure 1b). *C. albicans* survived up to 12.5 mg/mL thus having an MIC of 25 mg/mL while *S. cerevisiae* survived up to 25 mg/mL thus having an MIC of 50 mg/mL (Table 2). The zone of inhibition produced against the

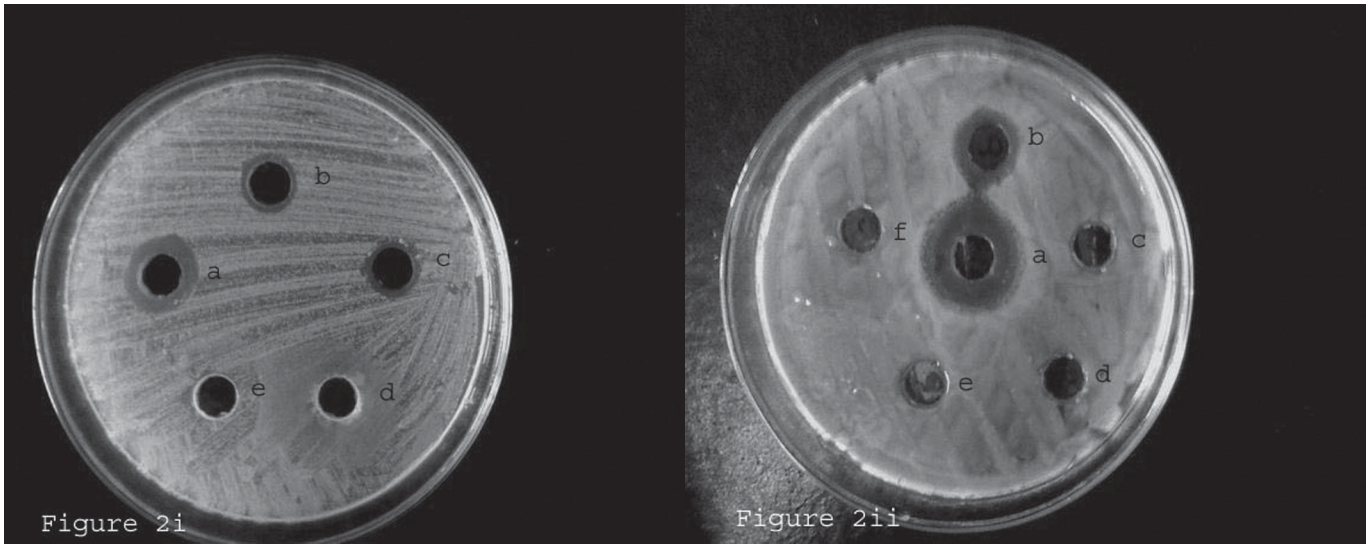

Fig. 2. MIC shown by acetonic extract of *P. cubeba* fruits against *C. albicans* (i) and *S .aureus* (ii) determined by modified agar well diffusion method using twofold serial dilutions of the extracts: 50 mg/mL (a), 25 mg/mL (b), 12.5 mg/mL (c), 6.25 mg/mL (d), 3.125 mg/mL (e) and 1.56 mg/mL (f).

Table 2. MIC of *P.cubeba* and *P.longum* fruit extracts against oral pathogens on specific media for each microorganism determined by modified agar well diffusion method.

Solvent extracts	Minimum Inhibitory Concentration (mg/mL)							
	S.mutans	*S.aureus*	*C.albicans*		*S.cerevisiae*			
	P.c.	*P.l.*	*P.c.*	*P.l.*	*P.c.*	*P.l.*	*P.c.*	*P.l.*
Acetone	50	-	25	-	12.5	25	12.5	50
Methanol	50	-	25	-	25	25	12.5	50
Ethanol	50	-	25	-	25	25	12.5	50

(-) = no activity, *P.c. = Piper cubeba, P.l. = Piper longum.*

bacterial pathogens by the three solvent extracts of *P. longum* fruits was mild that ranged between 10 and 11.31 mm without much variation in the different solvents activity (Table 1).

However, the bacteria were found to be comparatively resistant as they survived up to 50 mg/mL, thus having an MIC of 100 mg/mL. The fruit of *P. longum* contains a large number of alkaloids and related compounds, the most abundant of which is piperine, together with methyl piperine, pipernonaline, piperettine, asarinine, pellitorine, piperundecalidine, piperlongumine, piperlonguminine, retrofractamide A, pergumidiene, brachystamide-B, a dimer of desmethoxypiplar-tine, N - isobutyl-decadienamide, brachyamide- A, brachystine, pipercide, piperderidine, longamide, dehydropipernonaline piperidine and tetra hydro piperine[43]. Piperine, an alkaloid in the fruits of *P.longum* is responsible for the pungency of long pepper and has been shown to possess antiinflammatory, antiamoebic, antiasthmatic, anticonvulsant and antibacterial activities[44-45]. Thus, the antibacterial and antifungal activity of the fruits of *P. longum* may be due to the presence of the alkaloid piperine.

It may, therefore, be concluded from the above investigation that the crude extracts obtained from the fruits of the two *Piper* spp. may be used to treat oral fungal species, especially *C. albicans*, as they produced larger inhibition zones than the antifungal drugs often used to treat these pathogens. However, isolation of pure compounds and their toxicological analysis and clinical investigation in animal models are to be made before their trials on human.

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