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Comparative assessment of antimicrobial activity of five extract of *P. longum* and *P. nigrum* against *B. brevis, P. thailandensis, E. aerogenes* and *B. anthracis*

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ABSTRACT

In the present study the four candidate microbes *B. brevis, P. thailandensis, E. aerogenes* and *B. anthracis* were isolated from soils samples and were identified through various biochemical tests. The PCR amplification and sequencing of 16s rRNA gene was also performed for accurate identification of candidate microbes and showed up amplicon size of 718bp ,453bp, 581bp & 499bp respectively on agarose gel electrophoresis and sequencing. The antimicrobial activities of five different extract of *P. longum* and *P. nigrum* fruits have been investigated on the candidate microbes. *P. longum* & *P. nigrum* showed maximum antimicrobial activity against *E. aerogenes* and *B. brevis*, respectively in methanolic and isopropanolic extracts form correspondingly. *P. thailandensis* and *B. anthracis* were highest inhibited by *P. longum* in methanolic & isopropanolic extract form respectively, while by *P. nigrum* in isopropanolic extract form. In general, *Piper longum* demonstrated highest antimicrobial activity in methanolic extract form while *P. nigrum* in isopropanolic extracts form. This study emphasize that *Piper longum* and *P. nigrum* are enormously vital as natural antimicrobial agent or as it adjuvant and in microbiologically secure foods

Keywords: *P. longum, P. nigrum PCR amplification, 16s rRNA gene, Antimicrobial activity.*

INTRODUCTION

In the last three decades, pharmacological industries have produced a large number of new antibiotics (Al-Juraifani, A.A., 2011). Due to chaotic use of these antimicrobial drugs, rate of resistances in human pathogenic microorganisms has increased dramatically (Monroe and Polk, 2000; Parekh and Chanda, 2007; Cohen, M.L., 1992). Thus, especially in immunocompromised patients, the occurrence of high morbidity and mortality has been pragmatic with infection of these bacteria (Del Toro, M.D. , 2006; Driscoll, J.A., S.L. Brody and M.H. Kollef, 2007). These limitations and high cost of the drugs available today, up thrust the call for new natural chemotherapeutic substitutes from medicinal plants to eradicate the infections caused by drug resistant microbes and to ease the damage caused by such synthetic antibiotic.(Bocanegra-Garcia, V. , 2009; Giamarellou, H., 2006).

Natural products from plants have been used from ancient time to promote healthier human life, especially with advancement in natural therapies in the last decade (Gislene , 2000; Turgis M , 2008). Antimicrobial properties of plant products has been deeply emphasized for a potential application in food industry in order to prevent microbial growth (Hatha , 2006; Agaoglu , 2007; Gutierrez J, 2008; Holley RA, Patel D.2005; Du W-X, 2009; Sandasi M, 2008). Spices are not only been used for aroma and flavor of the foods but also for their antimicrobial properties (Nanasombat ,2002; Du W-X ,2011; Kivanç 1991;

Jansen , 1987; Shelef , 1980; Aureli , 1992; Conner, 1993). Extensive research has investigated the potential food applications of spices, in food products and animal feeds, for natural antimicrobial agents against foodborne pathogens (Tajkarimi M, 2011; Srinivasan D ,2001; Du W-X, 2011; Dorman H, Deans S 2008; Burt S. 2004; Singh A, 2003; Aktug and Karapinar, 1986; Raybaudi-Massilia RM 2006; Nanasombat S, and Lohasupthawee P., 20005). Spices and their derivative products and extracts, extensively contributes in foods and beverages industries (Praveen and Nazia, 2006; Sakandamis , 2002; Outtara , 1997; Arora and Kaur 1999).

Thus an increasing interest, of the scientific communities, in finding natural antimicrobials for application in food products to prevent or inhibit microbial growth and extend shelf life have been noticed (Gutierrez J , 2008; Lopes-Lutz D, 2008; Angioni A, 2004; Fattouch S 2007; Lanciotti R 2004).

Long pepper (*Piper longum Linn.*) is found both wild as well as cultivated, throughout the hotter parts of India. Black pepper (*Piper nigrum)* is native to southern Thailand and Malaysia, its most important habitat is the tropical regions of India. Black pepper has many medicinal properties like it is used to treat vertigo, asthma, chronicindigrstion, colon, toxins, obesity, sinusitis, congestion, fever, paralytic, arthritic disorders and also advised in diarrhoea and cholera (Shashidhar NS 2002; Ravindran PN 2000; Dorman HJ and Deans SG, 2000).

The present study was conducted to identify four pathogenic microbes from soil and to evaluate the antimicrobial activities of five different extracts of Long pepper Black pepper on the candidate isolated microbes.

MATERIALS AND METHODS

Collection of sample materials:

Long pepper and black pepper fruits were purchased from local fruits vendors for spice collection. After collection, the spices were shade dried at room temperature (32 - 35ºC) to constant weight over a period of 5 days. 400 g of each of the plant parts were powdered using an electric blender. The powder was transferred and stored in air tight closed containers.

Preparation of extracts:

Extracts were prepared in order to study their antimicrobial activity. Aqueous, ethanolic, methanol, isopropanol and chloroform extract of each of the long pepper and black pepper of different spice fruits was prepared by soaking 50g of the material in various solvents for 72 h with constant stirring at an interval of 24 h. Finally, the extracts were filtered through Whatmann filter (paper no. 1) and the filtrate was collected and incubated in water bath to obtain the crude extract (Alade and Irobi, 1993).

Identification Of Candidate Microbes:

Isolation of Bacterial Strains:

Two soil samples were randomly collected from different areas of waste land area in Lucknow, U.P. (India) region. Such samples were placed in separate sterile polythene bags and stored in a refrigerator at 4 °C till use.

The Luria Bertani Agar (LBA) and Nutrient Agar media (NAM) were prepared following the manufacturer's instructions for isolations. Using apposite isolation technique (Ali and Naseem, 2011; Beishir, 1991; Hampton, 1990), inoculation of bacteria was carried out under aseptic conditions. Inoculated petriplates were incubated at 37°C for 2-3 days. Different single colonies from two different plates were sub-cultured for purification. After 24 hours of incubation at 37°C, single colonies were streaked on four fresh media plates.

Biochemical Tests

Following etiquette as per Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974; Holding and Colle, 1971; Sneath, 1986; Taiwo and Oso, 2004) the four strains were isolated from different soils samples and were identified by various conventional biochemical tests. They are Gram reaction, Methyl-red test, Voges Proskauer tests, mannitol test, Catalase test and urease test.

Identification of Isolated Bacteria by PCR Amplification & Sequencing of 16S rRNA Gene

The PCR amplification of the DNA coding for 16s rRNA is the most powerful tool to identify the unknown bacteria. The 16s rRNA gene is amplified using the Polymerase Chain Reaction (Mullis, 1990), and the purified amplified product was outsourced for sequencing and the final sequence obtained was compared with the sequence obtained from the NCBI database.

Genomic DNA Isolation

Genomic DNA was extracted from four different cultures according to the following method. In four flasks, 50 mL LB broth was prepared and inoculated with different single bacterial colony and grown until an OD_{600} of 0.5–1.5. Bacterial cells were collected by centrifugation for 10 min at 5000 rpm, at 4°C. Bacterial Genomic DNA isolation kit (Qiagen, USA) was used

to isolate genomic DNA from each bacterial sample. The quantity and quality of isolated genomic DNA is estimated using Nanodrop spectrophotometer and finally stored at -20°C for further use.

Amplification of the 16s rRNA Gene of candidate microbes

The PCR was set up in 25 µl reaction volume. Based on initial trial, the reaction mixture was optimized as follows, 60ng of purified DNA, 2.5 µl of 10 X Assay buffer (100 mM Tris- HC1, pH 9.0, 15 mM MgC12, 500mM KC1 and 0.1% gelatin), 10 pm of forward and reverse primer, 0.20 mM of dNTP mix, 1U Taq DNA polymerase and autoclaved milliQ water to make up the volume. The primers used were designed by DNAStar software using sequences from NCBI database, Table 1.

Organism	Forward primer 5' – 3'	Reserve primer 5' – 3'	NCBI Accession No.
S1	cgggaggcagcagtagggaatt	caaccatgcaccacctgtcacc	AB101593
S2	gtcgagcggagttgatggagg	ggctttcttctcaggtaccgtca	AB265205
S3	ggcctaacacatgcaagtcgagc	ccaggttgagcccggggatttc	AJ251468
S4	atgcaagtcgagcgaatggattaag	ccacctacgtattaccgcggctgctg	AY138376

The cycling conditions contained an initial denaturation at 94 °C for 5 min followed by 35 cycles of 55 sec denaturation at 94 °C, 55 sec annealing at 62 °C and 60 sec elongation at 72°C and final elongation for 7 min, for micorobe *Brevibacillus brevis,* whereas annealing condition for microbe *Paenibacillus thailandensis, Enterobacter aerogenes and Bacillus anthracis* 50 sec annealing at 60 °C, 55 sec annealing at 58 °C and 50 sec annealing at 63 °C respectively. After the reaction, tubes with PCR products were held at 4 °C until further analysis. The products of PCR amplification were confirmed on a 1.6% agarose gel containing ethidium bromide (0.5 µg/mL) in 1XTAE buffer (Tris–Acetate–EDTA buffer) and visualized by UV transillumination. The PCR product was gel purified and subjected for sequencing (outsourced). The nucleotide sequence so obtained was compared with available sequences from the NCBI database and the candidate bacterial sequence was finally confirmed.

Evaluation of Antimicrobial activity

The candidate bacteria were grown in nutrient agar medium/broth. Antimicrobial activity of different extracts was measured using well diffusion method (Sawhney and Singh, 2000). For fresh culture, 25 ml of nutrient broth was taken in four sterilized test-tubes and inoculated with different candidate bacterium and incubator for 18-20 h. Milli Q water was used as negative control. 1ml of freshly prepared inoculums of different bacterial cultures was introduced in freshly prepared different sterilized molten medium plates. After media were solidified, the holes were made by cork borer (5 mm) and each well was filled with 50 µl of plant extract of both Long pepper and black pepper fruits. Finally, the bacterial culture plates were incubated for 24 h at 37°C to determine the zonal inhibition and measured in mm.

RESULTS AND DISCUSSION

In the present investigation, various results reveal that the different extracts of candidate fruits has great potential antimicrobial activity against candidate microbes.

Collection of fruit material:

The candidate fruits were purchased from local fruits market, and were shade dried at room temperature (32 - 35ºC) and coarsely powdered and stored in closed containers & in 4ºC temperature.

Preparation of extracts:

The five extracts were prepared in order to study the antimicrobial activity, namely aqueous, ethanolic, methanolic, chloroform and isopropanol extract of each candidate fruits and stored for further use.

Identification Of Candidate Microbes:

Biochemical Tests

The four bacteria isolated from different soils samples were identified by various conventional biochemical tests. The result of these biochemical tests revealed the probability of following four microbes, B. brevis, P. thailandensis, E. aerogenes and B. anthracis. The results of biochemical test are summarized in Table 2.

Test	Sample S1	Sample S2	Sample S3	Sample S4
Gram reaction	-	+	+	+
VP test	+	+	+	+
Methyl red	-	+	+	+
Catalase	+	+	+	+
Urease activity	+	+	+	+
Mannitol	-	-	-	-
Probable identify	B. brevis	P. thailandensis	E. aerogenes	B. anthracis

Identification of the Isolated Bacteria by PCR Amplification & Sequencing of 16S rRNA Gene

The genetic based identification of microbes through PCR based amplification and sequencing of 16s rRNA gene is most accurate, sensitive and potent tool for identification of microbes. The genomic DNA from four different pure cultures was isolated and visualized on 0.8 % agarose gel electrophoresis (Figure 1a). The good quality DNA was further used in PCR amplification reaction.

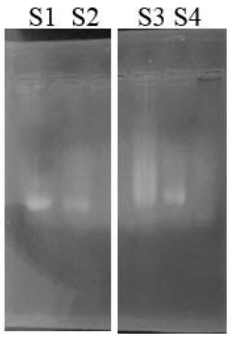

Figure 1a. Genomic DNA extracted from four pure cultures of candidate microbes

The 16S rRNA gene specific for four candidate microbes was amplified using specific primers Table-1. In order to ensure the amplification of specific fragment with higher yield, the PCR protocol was optimized with respect to reaction conditions as well as cycle parameters as mentioned in the materials and methods. Following PCR, the amplicons were checked using agarose gel electrophoresis. As expected a single and specific band were amplified from the DNA of respective candidate microbe. The amplicon size were 718bp, 453bp, 581bp & 499bp for samples of S1 (Brevibacillus brevis), S2 (Paenibacillus thailandensis), S3 (Enterobacter aerogenes) & S4 (Bacillus anthracis) respectively (Figure 1b).

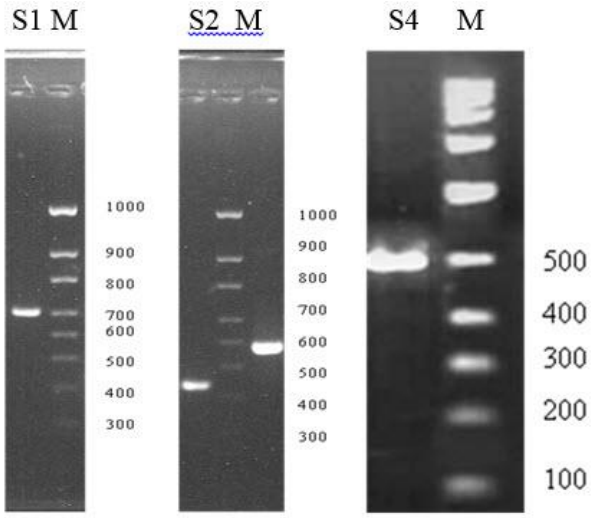

Figure 1b. PCR amplified product 718bp, 453bp, 581bp & 499bp of 16s rRNA gene of S1(*B. brevis*), S2(*P. thailandensis*), S3(*E. aerogenes*) & S4(*B. anthracis)* respectively, M = 100bp ladder

The four PCR products were gel purified and were sequenced (outsourced). The 5'-3' sequence of 16S rRNA gene of the respective microbes is shown in Figure 2. The sequences were BLASTED on NCBI website for sequence confirmation. The Sequencing confirmed the size of the amplified fragments to be 718bp ,453bp, 581bp & 499bp for samples of S1 (Brevibacillus brevis), S2 (Paenibacillus thailandensis), S3 (Enterobacter aerogenes) & S4 (Bacillus anthracis) respectively.

B. brevis 718 bp

Cgggaggcagcagtagggaattttccacaatggacgaaagtctgatggagcaacgccgcgtgaacgatgaaggtcttcggattgtaaagttctgttgttag
ggacgaataagtaccgttcgaatagggcggtaccttgacggtacctgacgagaaagccacggctaactacgtgccagcagccgcggtaatacgtaggtg
gcaagcgttgtccggatttattgggcgtaaagcgcgcgcaggcggctatgtaagtctggtgttaaagcccggggctcaaccccggttcgcatcggaaactg
tgtagcttgagtgcagaagaggaaagcggtattccacgtgtagcggtgaaatgcgtagagatgtggaggaacaccagtggcgaaggcggctttctggtctg
taactgacgctgaggcgcgaaagcgtggggagcaaacaggattagataccctggtagtccacgccgtaaacgatgagtgctaggtgttgggggtttcaata
ccctcagtgccgcagctaacgcaataagcactccgcctggggagtacgctcgcaagagtgaaactcaaaggaattgacgggggcccgcacaagcggtg
gagcatgtggtttaattcgaagcaacgcgaagaaccttaccaggtcttgacatcccgctgaccgctctggagacagagcttcccttcggggcagcggtgac
aggtggtgcatggttg

P. thailandensis 453bp

Gtcgagcggagttgatggaggtgcttgcacttctganggttagcggcggacgggtgagtaacacgtaggtaacctgcccataagaccgggataacattcg
gaaacggatgctaataccggatacgcaattctctcgcatgaggggattgggaaaggcggagcaatctgtcacttatggatggacctgcggcgcattagcta
gttggtgaggtaacggctcaccaaggcgacgatgcgtagccgacctgagagggtgatcggccacactgggactgagacacggcccagactcctacggg
aggcagcagtagggaatcttccgcaatgggcgaaagcctgacggagcaacgccgcgtgagtgatgaaggttttcggatcgtaaagctctgttgccaggga
agaacgcttgggagagtaactgctctcaaggtgacggtacctgagaagaaagcc

E. aerogenes 581bp

ggcctaacacatgcaagtcgagcggtarcacagagagcttgctctcgggtgacgagcggcggacgggtgagtaatgtctgggaaactgcctgatggagg
gggataactactggaaacggtagctaataccgcataacgtcgcaagaccaaagtgggggaccttcgggcctcatgccatcagatgtgcccagatgggatta
gctagtaggtggggtaatggctcacctaggcgacgatccctagctggtctgagaggatgaccagccacactggaactgagacacggtccagactcctacg
ggaggcagcagtggggaatattgcacaatgggcgcaagcctgatgcagccatgccgcgtgtatgaagaaggccttcgggttgtaaagtactttcagcgag
gaggaaggcgttaaggttaataaccttggcgattgacgttactcgcagaagaagcaccggctaactccgtgccagcagccgcggtaatacggagggtgca
agcgttaatcggaattactgggcgtaaagcgcacgcaggcggtctgtcaagtcggatgtgaaatccccgggctcaacctgg

B. anthracis 499 bp

atgcaagtcgagcgaatggattaagagcttgctcttatgaagttagcggcggacgggtgagtaacacgtgggtaacctgcccataagactgggataactcc
gggaaaccggggctaataccggataacattttgaaccgcatggttcgaaattgaaaggcggcttcggctgtcacttatggatggacccgcgtcgcattagct
agttggtgaggtaacggctcaccaaggcaacgatgcgtagccgacctgagagggtgatcggccacactgggactgagacacggcccagactcctacgg
gaggcagcagtagggaatcttccgcaatggacgaaagtctgacggagcaacgccgcgtgagtgatgaaggctttcgggtcgtaaaactctgttgttaggga
agaacaagtgctagttgaataagctggcaccttgacggtacctaaccagaaagccacggctaactacgtgccagcagccgcggtaatacgtaggtgg

Figure 2: Nucleotide sequence of 718bp, 453bp, 581bp & 499bp of 16s rRNA gene of S1 (*B. brevis*), S2 (*P. thailandensis*), S3 (*E. aerogenes*) & S4 (*B. anthracis)* respectively.

Antimicrobial activity

The Piper longum possessed maximum antimicrobial activity against B. brevis, P. thailandensis, E. aerogenes in the methanolic extract form while maximum against B. anthracis in isopropanol extract form. While lowest antimicrobial against B. brevis, P. thailandensis, E. aerogenes and B. anthracis was demonstrated by aqueous, ethanol, isopropanol and chloroform extract form respectively (Fig 3 and Table 3).

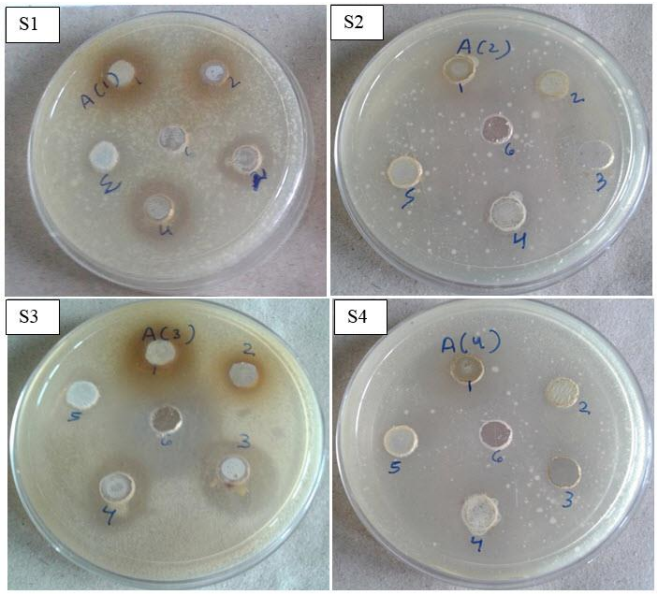

Figure 3. Zone of inhibition of different extracts of *Piper longum* against S1, S2, S3, & S4 micro-organism

Table 3. Antimicrobial activity of Piper longum extracts against micro-organism of S1, S2, S3 and S4. Extract form: A= Methonol, B= Chloroform, C= Ethanol, D=Isopropanol, E=Aqueous and F= Water (control)

Organism	Piper longum: Diameter of zone of inhibition (mm) Extract					
	A	B	C	D	E	F
S1	10	7	8	9	6	0
S2	11	7	6	8	8	0
S3	16	10	14	8	10	0
S4	11	7	8	12	10	0

The Piper nigrum possessed maximum antimicrobial activity against B. brevis, P. thailandensis, E. aerogenes and B. anthracis in the isopropanol extract form. While lowest antimicrobial against B. brevis, P. thailandensis and B. anthracis, was demonstrated by chloroform extract form and lowest against E. aerogenes aqueous extract form. (Fig 4 and Table 4).

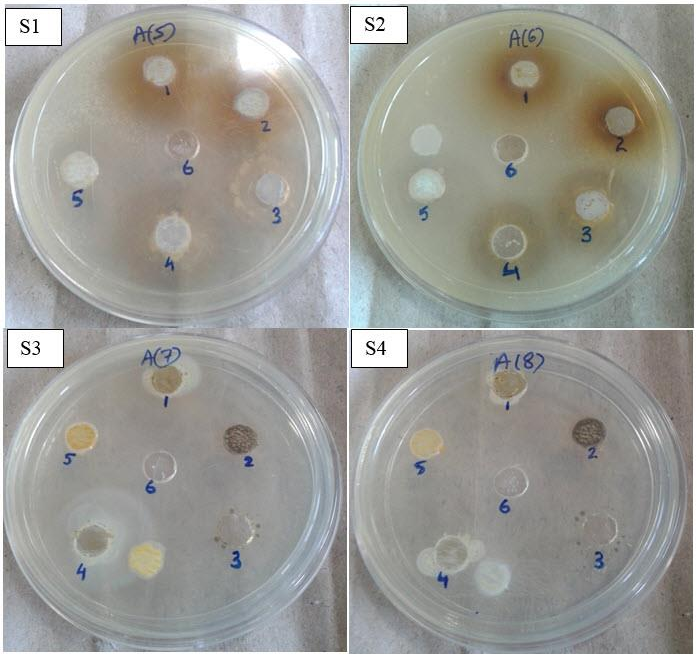

Figure 4. Zone of inhibition of different extracts of P*iper nigrum* against S1, S2, S3, & S4 micro-organism

Table 4. Antimicrobial activity of Piper nigrum extracts against micro-organism of S1, S2, S3 and S4.

Organism	Piper nigrum: Diameter of zone of inhibition (mm) Extract					
	A	B	C	D	E	F
S1	7	6	10	13	7	0
S2	7	6	8	10	9	0
S3	9	7	6	10	5	0
S4	9	6	7	10	7	0

This study strongly evidence that the maximum antimicrobial activity against any specific microbes varies with the different solvent-extract form. A more comprehensive study need to be done to compile a data for all spices to identify the best antimicrobial extracts form as broad spectrum as well as individual microbes specific, so as to be used as potential food additives or as natural antimicrobial adjuvant to assure the microbiologically secure foods production.

CONCLUSION

From this study, it can be concluded that the peel and pulp of candidate citrus fruits encourage a very high potential to be used as natural antimicrobial agents against studied microbes in comparison to gentamicin. Further research may also be done to study the antimicrobial properties of these citrus fruits against other microbes as well as in comparison to other synthetic antibiotics also. It can also be concluded that the peel and pulp of candidate citrus fruits are valuable for human consumption and health. Further, this study put forward a new insight towards formulation of new & effective antimicrobial drug and can be used as a powerful natural antimicrobial stabilizer for food products and as nutritional supplement.

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