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ANTIBACTERIAL ACTIVITY OF PIPPALI PROTEINS (*PIPER LONGUM*)

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ABSTRACT

Pippali (*Piper longum*) belongs to *Piperaceae*, cultivated for its fruit widely used medicine for diseases of the respiratory tract, viz. cough, bronchitis, asthma, etc. as counter- irritant, analgesic and as anti-inflammation. Here in we studied the antibacterial property of *Piper longum* protein isolated from hot water extract of *Piper longum*. The protein was isolated from *Piper longum* by 65% ammonium sulphate precipitate and followed by dialysis using molecular cutoff membrane. The proteins were examined for their antioxidant and antibacterial activity. The protein at 25 µg concentration showed about 59% inhibition against DPPH. The proteins were checked for their antibacterial activity against human pathogenic bacteria's like *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas, Salmonella typhimurium, Streptococcus Sps, Staphylococcus aureus, Vibrio cholera.* MIC value of antioxidant protein ranged from 90-106 µg/ml. MIC was about 12-15 µg/ml. Streptomycin was used as a standard antibiotic. These results show that, the antioxidant protein from Turmeric is an effective antioxidant as well as antimicrobial agent.

KEYWORDS

Pippali, *Piper longum*, Proteins and Antibacterial.

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INTRODUCTION

Natural dietary compounds are the main source of therapeutic agents to deal with communicable and non-communicable diseases[1]. The microbiologists are in search of bioactive sources which could be developed as promising drugs for treatment of infectious diseases and other biomedical challenges including drug resistance among various infectious microbes[2]. The genetic ability of pathogenic bacteria to develop resistance against frequently used antibiotics is a medical problem and worldwide challenge, posing a big threat to human society[3-5].

Antibiotics kill or inhibit the growth of microbes such as bacteria, fungi, protozoa's and viruses. Antimicrobial drugs either kill microbes (microbicidal) or prevent the growth of microbes (microbistatic). The toxicity to humans and other animals from antibiotics is generally considered to be low whereas prolonged use of antibiotics shows a negative impact on health. Sometimes, antibiotics are associated with adverse effects including hypersensitivity, immune-suppression and allergic reactions and also emergence of antibiotic resistant microbial strain had been a concern. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases form various natural sources such as medicinal plants with lesser side effects. Medicinal plants, as well as tribal and folk medicinal uses are of great concern among the natural product researchers, for getting new bioactive sources[6,7]. Pippali is the dried ripe fruits; Pippalimulam is the roots of this plant[8]. The fruit contains biochemicals like volatile oil, *piperine* and *piperlonguminine*, N- isobutyldeca-trans-2-trans-4-dienamide and a terpenoid substance[9]. It was reported that, crude extract of Long pepper exhibits antibacterial activity; its isolates are active against Gram positive bacteria and moderately active against Gram negative bacteria. Each isolate is highly active against at least one particular species of bacteria: piperlonguminine against *Bacillus subtilis* and piperine against Staphylococcus aureus[10,11]. In the present study, we have checked for the antioxidant and anti-bacterial activity of *Piper longum* proteins.

MATERIALS AND METHODS

Long pepper (*Piper longum*) was obtained from the local market of Mysore, Karnataka, India, BHA, α-tocopherol, Ascorbic acid were purchased from Sigma Aldrich Co, (St. Louis, USA), Other chemicals unless otherwise mentioned were procured from Merck India Co, Mumbai. Pure clinical isolates of human pathogenic bacteria, *Escherichia coli*, *Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas, Salmonella typhimurium, Streptococcus Sps, Staphylococcus aureus, Vibrio cholera* were obtained from Microbiology department, Adichunchanagiri Institute of Medical Sciences (AIMS), B.G. Nagara, Karnataka, India.

Isolation of proteins from *Piper longum*

Ten grams of *Piper longum* powder was added to 150 ml boiling double distilled water, vortexed at ambient temperature for two hours. It was centrifuged at 10,000 rpm at 4^{o}C for 20 min. The supernatant was filtered through Whitman No.1, filter. Further, the supernatant subject to 55% ammonium sulphate precipitation and vortexes for overnight. Centrifuged and the precipitated proteins was separated. The precipitate was subjected to dialysis using 2kDa molecular cut off membrane against double distilled water for 76 hours at a interval of 6 hours each. Now the obtained proteins were subjected to proximate analysis.

Proximate analysis

The proximate analysis such as protein, total sugars, polyphenols, flavonoids, ascorbic acid, α-tocopherol was done.

Estimation of Proteins

The protein content of the both extracts was estimated according to Bradford's method[12] using bovine serum albumin as standard. Aliquots were made up to 0.1ml with distilled water and 0.9 ml of Bradford's reagent was added. The total protein content was calculated using the standard curve.

Estimation of total sugar

The total sugar concentration Long pepper extract was estimated by Dubois method[13] and Dextrose used as standard. Different aliquots were made up to 1ml with distilled water followed by the addition of 1ml of 5% phenol and 5ml of concentrated sulphuric acid and were read at 520nm. The sugar concentration was calculated accordingly.

Estimation of total phenolic

Total content of phenolic was determined by the method of Folin-ciocalteau reaction using Gallic acid as standard[14]. The extract and the standard Gallic acid were dissolved in 0.5ml of water and were mixed with 500µl of 50% Folin-ciocalteau reagent. The mixture was then allowed to stand for 10min followed by the addition of 1.0ml of 20% Sodium carbonate. Incubated at 37^{0}C, 10 min and the absorbance of the supernatant were read at 730nm. The total phenolics content was

expressed as Gallic acid equivalents in milligrams per gram of powder.

Estimation of flavonoids

Total flavonoid content was determined colorimetrically using spectrophotometer[15]. Standard calibration curve was prepared using Quercetin. 10mg of Quercetin was dissolved in 80% ethanol and then diluted solutions were separately mixed with 1.5ml of 95% ethanol, 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415nm by using proper controls. The total Flavonoid content was calculated accordingly.

Estimation of α-tocopherol

α-tocopherol estimation was carried out according to Kivcak and Mer[16]. 20µl-100µl of standard α-tocopherol solution and 20 and 40µl of the hexane extracts was used for the estimation. Volume was made up to 3ml using chloroform, 1 ml of 2, 2-dipyridyl, and 1 ml of $FeCl_3$ solution, Incubated at 37^0C for 15 minutes, and the absorbance of the reaction mixture was read at 520nm, concentration was calculated accordingly by using the standard graph.

Estimation of Ascorbic

Ascorbic estimation was carried out according to Sadasivam S, Manickam[17]. Different concentrations (0-100µg) of hexane extracts were taken along with standard ascorbic acid. A drop of thiourea solution and 1ml of 2,4dinitrophenyl hydrazine reagent was added to each tube and the volume as made up to 100µl with 4% oxalic acid and incubated at 37°C for 3 hours. Then tubes cooled on ice water and 5ml of 85% sulphuric acid was added to each tube. Mix the reaction mixtures thoroughly. The orange color developed was read against a reagent blank at 540nm. The concentration was calculated on the basis of the standard curve.

ANTIOXIDANT ACTIVITY

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Shimada et al. with minor modifications[18] *Piper longum* at a concentration of 25µg each was mixed in 1 ml of freshly prepared 0.5 m MDPPH ethanolic solution and 2 ml of 0.1 M acetate buffer pH 5.5. The resulting solutions were then incubated at 37°C for 30 min and measured spectrophotometrically at 517 nm. BHA (400 µM), Ascorbic acid (400 µM) and α-tocopherol (400 µM) was used as positive control under the same assay conditions. Negative control was without any inhibitor or *Piper longum*. Lower absorbance at 517 nm represents higher DPPH scavenging activity. The % DPPH radical scavenging activity of extracts of *Piper longum* was calculated from the decrease in absorbance at 517 nm in comparison with negative control.

Antimicrobial activity and minimum inhibition concentration of *Piper longum* proteins

The isolated protein was screened for its antibacterial activity by paper disc method[19]. This was confirmed by the inhibitory effect on bacterial growth as reflected by inhibition zone compared to known antibiotics. Using petriplates of size 90mm×15mm containing 15ml of nutrient agar. Suspension of different test bacteria such as *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas, Salmonella typhimurium, Streptococcus, Staphylococcus aureus, Vibrio cholerae* was swabbed over the medium and autoclaved Whattmann filter paper disc (5mm in diameter) were placed equidistantly from the rim of the plate that were pretreated with antibiotics aseptically. Aliquots of different concentrations were applied to a disk and sterile distilled water was added to the disc as a negative control. Streptomycin, an antibacterial agent (1:10 dilution) was used as the positive control. The bacteria cultured Petri plates were incubated at 36 ± 2°C for 48 hours. A transparent ring around the paper disc signifies Inhibition zone which depicts the antibacterial activity. The inhibition zone was measured in mm from the edge of the disc to the inner margin of the surrounding pathogens.

The minimum inhibitory concentration (MIC) was determined by serial dilution[20] of crude proteins of *Piper longum* in the broth medium, with different concentrations. Plates were incubated for 24 hours at 37^0C for bacteria and 48 hours at 24^0C for yeast

cultures. MIC was recorded as lowest extract concentration demonstrating no visible growth in the broth. All the plates were done in triplicates.

RESULTS AND DISCUSSION

The proteins were isolated as explained in materials and methods. In brief, 55% ammonium sulphate precipitation was done to precipitate the proteins.

Piper longum proteins were isolated from double distilled water extract of Piper longum as explained in methods. The excess ammonium sulphate was removed by dialysis against water using molecular cut off membrane at 20°C. The isolated dialyzed proteins were subjected to proximate analysis to estimate the phytochemicals presents. The analysis result showed in TableNo.1 that, the dialyzed protein extract rich in proteins and contains very negligible amount of free sugars and flavonoids. As discussed in materials and methods, the proteins were analyzed for their antioxidant activity by DPPH radical scavenging activity where, lipid soluble α-tocopherol, water soluble Ascorbic acid and BHA were used as standard antioxidants at a maximum dosage of 400µM and proteins of piper longum used at a dosage of 25µg. Table No.2 showed that, α-tocopherol, Ascorbic acid and BHA showed a % inhibition of DPPH radicals 64%, 55% and 52% respectively. Whereas, the piper longum proteins showed a inhibition of 59%. This means, Long pepper proteins have good antioxidant activity when compared to standards. Further, the antibacterial activity of Piper longum proteins tested against many human pathogenic bacterial strains, among all, it is found to be more effective against the selected bacteria's. Streptomycin was taken as positive control. Table No.3 shows that, MIC values of Piper longum proteins against selected bacterial strains and standard antibiotic Streptomycin. The above results indicate that, proteins of Piper longum is a good antioxidant and also anti-bacterial agent. It is effective at lower concentrations and also non-toxic. It shows a broad spectrum of anti-bacterial activity.

Table No.1: Phytochemical Analysis of Dialyzed *Piper Longum* Proteins

S.No	Tests	Dialyzed (g %)
1	Protein	**0.108**
2	Ascorbic acid	Nil
3	Sugars	0.002
4	Polyphenols	Nil
5	Flavonoids	0.003
6	α-tocopherol	Nil

The results represents mean ± S.D (n = 3)

Table No.2: Antioxidant activity of *Piper Longum* Proteins in Comparison with standard antioxidant

S.No	Antioxidants	Concentration	% of DPPH Radical Scavenging
1	α - tocopherol	400 µM	64 %
2	Ascorbic acid	400 µM	55 %
3	BHA	400µM	52%
3	Proteins of *Piper longum*	25 µg	59%

The results are mean ± S.D (n = 6)

Table No.3: Minimal Inhibitory Concentration (Mic) of *Piper Longum* Proteins against Different Microbial Strains in Serial Dilution Method

S.No	Bacterial Strains	MIC of Peak I (µg/ml)	Positive Control Streptomycin (µg/ml)
1	*Escherichia coli*	106 ± 0.5	15 ± 0.5
2	*Klebsiella pneumoniae*	101 ± 0.5	13 ± 0.5
3	*Proteus vulgaris*	90 ± 1	12 ± 1
4	*Pseudomonas*	92 ± 0.5	12 ± 1
5	*Salmonella typhimurium*	110 ± 2	13 ± 0.5
6	*Streptococcus sps*	93 ± 1	12 ± 1
7	*Staphylococcus aureus*	92 ± 0.5	15 ± 0.5
8	*Vibrio cholerae*	98 ± 0.5	12 ± 1

CONCLUSION

The proteins of *Piper longum* can be effectively used as antioxidant as well as antibacterial agent. Further study need to be done in purifying the proteins and characterizing it.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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