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Antioxidant and *In Vitro* Antidiabetic Activity of Methanol Extract of *Piper cubeba* L.

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***Abstract*:**
Objective- *The study was carried out to evaluate invitro antioxidant and antidiabetic activity of methanol extract of medicinal plant Piper cubeba L.*
Methods- *In vitro antioxidant activity of various concentrations of plant extract (20-100 µg/ml) was carried out by using DPPH free radical scavenging assay. The reduced color intensity was measured by using UV visible spectrophotometer at 517 nm. The ascorbic acid was used as a reference drug. In vitro antidiabetic activity of various concentrations of plant extract was investigated by α-amylase inhibitory assay and glucose uptake in yeast cells assay.*
Results- *There was dose dependent percentage inhibition of DPPH free radical scavenging activity. The maximum 79.56% inhibition was observed at 1000 µg/ml and the IC50 value found at 58.75 µg/ml, indicating potent antioxidant action. The maximum 80.59% α-amylase activity inhibition was observed at concentration 250 µg/ml of plant extract. The standard drug acarbose exhibited 86.16% inhibition. Similarly, maximum 65.19% increase in glucose uptake by yeast cell was reported at 250 µg/ml of plant extract.*
Conclusion- *Methanol extract of fruits of medicinal plant Piper cubeba exhibited potential antioxidant and antidiabetic activity. This would attribute to presence of bioactive substances polyphenols and flavonoids.*

Keywords- *Antidiabetic; Antioxidants; Amylase; Free radicals; Yeast cells.*

Conflict of Interest- *The authors declare no conflict of interest. The authors alone are responsible for the writing and content of the paper.*

I. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder which is characterized by hyperglycemia, hypoinsulinaemia, polyphagia, polyuria, polydipsia, polyproteinuria, and loss of body weight that affects people of all ages across the world [1]. The prevalence of diabetes mellitus is continuously rising globally and India is becoming the diabetes capital of the world by accounting for nearly 20% of the world diabetic patients. It is estimated that, by 2030 about 79.4 million Indian people will be suffering from this disease [2, 3]. The various long term micro vascular and macro vascular complications of DM include retinopathy, neuropathy, nephropathy, heart attack, stroke, and peripheral vascular diseases [4]. The main types of DM are type 1, type 2 and gestational diabetes. The only therapy for type 1 diabetes is substitution of insulin but type 2 is controlled by various hypoglycemic agents such as sulphonylureas, biguanides, glinides etc. [5]. There is no single drug is available as complete remedy to diabetes. The conventional antidiabetic drugs acts either by preventing the breakdown of dietary carbohydrates into glucose or preventing the diffusion of the glucose through the intestinal membrane into circulation. The inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase is helpful in reducing postprandial blood glucose levels [6]. All the existing antidiabetic agents are having considerable unwanted effects and moreover these drugs are not safe during pregnancy condition [7]. Thus, in recent years herbal medicines have started to gain importance as a source of hypoglycemic agents.

Medicinal plants have been used for the treatment of various diseases since ancient times. Phytochemical constituents like saponin, phenols, flavonoids present in the plant showed potential antidiabetic and antioxidant activity [8]. More than 400 plants worldwide have been reported as beneficial in the treatment of diabetes but are lacking the proper scientific and medical evaluation. The medicinal plant *Piper cubeba* L. belongs to the family Piperaceae commonly known as 'Java pepper' is a popular medicinal plant which has been extensively used in many countries including India [9]. The fruit are used for the treatment of rheumatism, abdominal pain, asthma, diarrhea, dysentery, gonorrhea, enteritis and syphilis [10] and has also been proved to have an inhibitory action on hepatitis C virus protease [11]. In addition, the extract has been reported to potential antiestrogenic, anti-inflammatory, nephroprotective, cytotoxic, antiparasitic and antimicrobial action [12].

As, active research has been performed on traditionally available medicinal plants for the discovery of new antidiabetic drug as an alternative for synthetic drugs, the current research was undertaken to evaluate *in vitro* antioxidant and antidiabetic potential of *Piper cubeba* L.

II. MATERIALS AND METHODS

Chemicals and Instruments

DPPH (Sigma-Aldrich, USA), Dinitrosalicylic acid (DNS), α –amylase (S.D. Lab. Chem. Mumbai), metformin (Franco-Indian Pharm. India), ascorbic acid, acarbose (Sigma Chem. Co., USA), baker's yeast, UV-visible spectrophotometer (Shimadzu 1800), colorimeter (Equiptronics, India). All the reagents and solvents were of analytical grade.

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Plant Material and Preparation of Extract

The dry fruits of *Piper cubeba* L. were collected from local market (Dorle & Sons Ayurveda, Kolhapur, Maharashtra, India) and herbarium was authenticated (Auth.15-118) at Agharkar Research Institute Pune, India and the specimen was deposited in Department of Biodiversity & Palaeobiology.

The 500 g of dry fruits were grinded to fine powder (sieve 44) and was subjected to methanol extraction by using continuous Soxhlet's apparatus for 48 h. The extract was filtered through Whatman filter paper (No 1) and the residue obtained was carried to rotary evaporator under reduced pressure for further dryness. The powdered extract was stored in air tight container.

DPPH Free Radical Scavenging Assay

The *in vitro* free radical scavenging activity of methanol extract of *Piper cubeba* was determined by using DPPH according to method [13] with slight modification. Briefly, 0.1 mM solution of DDPH in methanol was prepared and 1 ml of this solution was added to 3 ml of control (an equivalent amount of methanol) and test solutions at different concentrations (20, 40, 60, 80, and 100 µg/ml) in different test tubes. Thirty minutes later, the absorbance was measured at 517 nm by using UV-visible spectrophotometer. The percentage ability to scavenge the DPPH radical was calculated by using the equation:

$$\% \text{ DPPH scavenged} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control and At is the absorbance of the test sample. The IC50 value was obtained by linear regression analysis of the dose response curve plotted using % inhibition versus concentration. The ascorbic acid was taken as a reference standard [13].

In Vitro α-Amylase Inhibitory Assay

The 0.5 mg/ml enzyme solution was prepared by dissolving α -amylase in 20 mM phosphate buffer (pH 6.9). The colorimetric reagent is 3, 5 -di nitro salicylic acid (DNS) was prepared by dissolving 1 g of DNS, 30 g of sodium potassium tartarate and 20 ml of 2 N sodium hydroxide was added and made up to a final volume of 100 ml with distilled water. A 1% w/v starch solution was prepared in 20 mM phosphate buffer. Different concentrations of extract (50, 100, 150, 200, and 250 µg/ml) were prepared for inhibitory assay. Similar concentrations of acarbose solutions were made as a reference standard.

Briefly, α-amylase inhibitory assay was carried out by adding 1ml of enzyme solution to test tubes containing 1ml of various concentrations extract and incubated at 25°C for 10 min. After incubation, 1ml of starch solution was added to the mixture and further incubated at 25°C for 10 min. The reaction was then stopped by adding 2 ml of DNS color reagent and incubated in boiling water for 5 min. After cooling, the test tubes are diluted to 10 ml with buffer solution and the absorbance was measured on UV-visible spectrophotometer at 540 nm. Control, representing 100% enzyme activity was prepared in a similar manner, without extract. The experiments were carried out in triplicate. The % inhibition of α-amylase activity was calculated using the following formula:

$$\% \text{ Inhibition} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control and At is the absorbance of the test sample. From a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC50 value of the sample [14, 15].

In Vitro Glucose Uptake Assay in Yeast Cells

Briefly, the commercial baker's yeast was obtained and subjected to distilled water for repeated centrifugation (3000g, 5 min) until the clear supernatant fluid was appeared and a 10% (v/v) of the suspension was prepared in distilled water. Various concentrations of plant extracts (50, 100, 150, 200, and 250 µg/ml) were added to 1ml of glucose solution (5, 10 and 20 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µl of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2500 g, 5 min) and amount of glucose was estimated in the supernatant [16, 17]. Metformin was used as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the formula:

$$\% \text{ increase in glucose uptake} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control and At is the absorbance of the test sample. All the experiments were carried out in triplicates.

III. Results

The *in vitro* antioxidant activity of methanol extract of *Piper cubeba* was carried out by using DPPH scavenging assay. The extract showed dose dependent percentage inhibition of free radicals. The maximum 79.56% inhibition was observed at 1000 µg/ml of plant extract while that of standard drug ascorbic acid showed 89.65%. The IC50 values were 58.75 µg/ml and 37.60 µg/ml for plant extract and standard drug respectively (Figure 1).

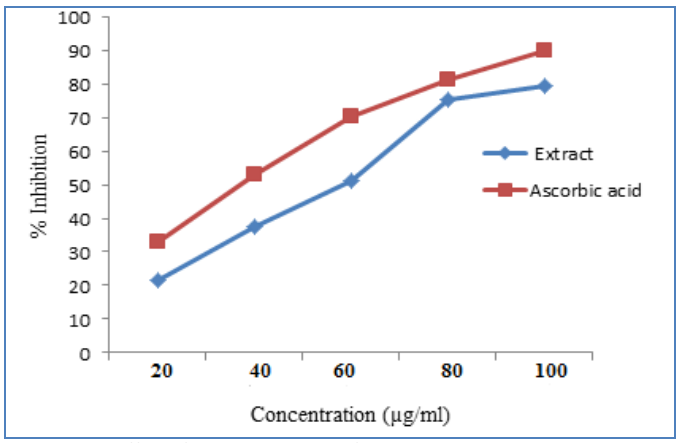

Fig. 1. Effect of methanol extract of *Piper cubeba* on DPPH radical scavenging assay.

The effect of methanol extract of *Piper cubeba* on α-amylase activity was shown in table I. The extract showed dose dependent percentage inhibition. The concentration 250 µg/ml of plant exhibited highest 80.59% inhibition of α-amylase activity and 86.16% inhibition was showed by

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standard drug acarbose at the same concentration. The IC50 value for plant extract and standard drug acarbose was found to be 85.50 µg/ml and 64.60 µg/ml respectively.

TABLE I. *In vitro* α-amylase inhibitory effect of methanol extract of *Piper cubeba* L.

Concentration (µg/ml)	% Inhibition of α-amylase activity	
	***Piper cubeba* extract**	**Acarbose**
50	38.76±2.65	42.67±1.87
100	51.73±1.46	58.91±1.23
150	63.14±1.98	69.43±0.99
200	71.43±2.01	78.54±1.48
250	80.59±1.75	86.16±1.62
IC50 value	85.50±1.29	64.60±0.75

Values expressed as mean of three replicates ± S.E.M.

Similarly, the *in vitro* glucose uptake activity was investigated in yeast cells of species Saccharomyces cerevisiae. The results showed that, there was a dose dependent increase in percentage uptake of glucose by yeast cells for plant extract as well as the standard drug metformin (Table II). Concentration of plant extract as well as metformin at 250 µg/ml showed maximum 65.19% and 81.16% increase in glucose uptake by yeast cells. The reported IC50 values were 162.20 µg/ml and 114.32 µg/ml for plant extract and metformin respectively.

TABLE II. Effect of methanol extract of *Piper cubeba* on % increase in glucose uptake by yeast cell.

Concentration (µg/ml)	% increase in glucose uptake	
	***Piper cubeba* extract**	**Acarbose**
50	19.46±3.15	24.77±1.67
100	31.77±2.43	38.81±1.29
150	42.34±1.08	59.43±1.99
200	53.49±2.11	76.24±1.41
250	65.19±2.38	81.16±1.62
IC50 value	162.20±1.18	114.32±2.27

Values expressed as mean of three replicates ± S.E.M.

IV. DISCUSSION

Excess production of free radicals induces variety of diseases such as diabetes, cancer, inflammation, ulcer, arthritis, and neurodegeneration [18]. The role of medicinal plants in disease prevention is attributed to antioxidant properties of their bioactive substances like phenols, flavonoids and proanthracyanidins. The antioxidant property of plants originates from capability of proton loss, chelate formation, and dismutation of radicals by bioactive substances [19]. DPPH method is widely used for determination of *in vitro* free radical scavenging activity several substances. The antioxidants react with the stable DPPH radical which is deep violet color and convert it to α-α diphenyl β-picrylhydrazine with discoloration. The decreased color intensity indicates the free radical scavenging potential. In the present study methanol extract of *Piper cubeba* exhibited high potential of DPPH free radical scavenging activity. The lower IC50 value would reflect greater antioxidant potential of sample. The plant extract has been already proved to high content of phenolics and flavonoids [20]. Thus, antioxidant ability of *Piper cubeba* may attributes to presence of phenolic and flavonoid content.

The carbohydrate metabolic disorder may causes various health problems including diabetes. Diabetes mellitus is mainly due to the lack of insulin secretion or action. The treatment of type-II diabetes includes the stimulation of insulin secretion, inhibition of breakdown of polysaccharides and disaccharides etc. The intestinal digestive enzyme α-amylase plays an important role in the carbohydrate digestion. [21]. A pancreatic α-amylase enzyme catalyzes dietary starch into a mixture of small oligosaccharides in gastrointestinal tract. Further, in presence of α-glucosidase enzyme these oligosaccharides degrades into glucose which then diffuses through the intestine wall into the blood stream, increasing postprandial blood glucose levels [22]. Hence, the inhibition of such carbohydrate digesting enzyme is an important tool in the evaluation of antidibetic activity. In the present study, methanol extract showed high potential inhibition of α-amylase activity in concentration dependent manner. So the plant extract might be used as starch blockers since it prevents or reduces the absorption of starch in to blood mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose, maltriose and other simple sugars. The presence of polyphenols and flavonoids in the plant extract not only capable of reducing oxidative stress but also of inhibiting carbohydrate hydrolyzing enzymes as their ability to bind with proteins.

In yeast glucose transport takes place through facilitated diffusion. The characteristic of the sugar transport system in the yeast is gaining a major attention for researchers. Yeast is able to utilize one or more sugars as their principal source of carbon and energy. Further, the sugar converts into ethanol [23]. Therefore inhibition of such yeast cell is a major tool for investigation of *in vitro* antidiabetic activity of several drugs and medicinal plants. In the present study, after the treatment of the yeast cells with the methanol extract of plant, the glucose uptake was found to increase in a dose dependent manner which amplifies antidiabetic potential.

V. CONCLUSION

The methanol extract of *Piper cubeba* proved a potential medicinal plant for treatment of diabetes. The *in vitro* antidiabetic action may be due to its antioxidant property. Further, an isolation and characterization of bioactive molecules is necessary to reflect underlying mechanism.

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