

In vitro therapeutic correlative studies of *Pandanus odoratissimus* flowers against diabetic foot ulcer-causing bacterial pathogens

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Abstract

Background: Diabetic foot ulcer (DFU) is a major secondary complication that arouses subsequently due to careless of patient in poor therapeutic management of chronic diabetes. This may sometimes lead to amputation of foot which creates mental stress and societal empathy among diabetic patients. Hence, the aim of the present investigation is formulated to evaluate and correlate the *in vitro* antioxidant efficacy, *in vitro* invertase inhibitory activity and *in vitro* antibacterial activity of *Pandanus odoratissimus* flower extracts. **Materials and Methods:** *In vitro* cupric ion reducing antioxidant capacity and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assays were performed to analyze the reducing power and radical scavenging property of *P. odoratissimus* flower extracts. Standard broth dilution and agar well diffusion assays were espoused for antibacterial activity. Similarly, DNS method is adopted to investigate the invertase inhibitory activity. **Results and Discussion:** The bacterial pattern identification studies carried out with the clinical isolates (pus and blood) of DFU patients have revealed the presence of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The methanol (12 ± 3 mm) and acetone (16 ± 4 mm) crude extracts possessed significant zone of inhibition against *E. coli*, and near satisfactory control was observed against *S. aureus* and *P. aeruginosa*. Significant dipicryl ion scavenging activity (DPPH) was recorded by methanolic ($90.7 \pm 2\%$) and aqueous extracts ($81.4 \pm 3\%$). Similarly, a satisfactory cupric ion reducing property was established by the methanolic (effective dosage [ED₅₀]: 455.3 ± 8 µg/ml) and aqueous extracts (ED₅₀: 452.14 ± 5 µg/ml). An effective *in vitro* invertase inhibitory activity ($62.5 \pm 3\%$) was recorded by methanolic extract. **Conclusion:** The studies proved a strong correlation exist between antioxidant power, invertase inhibition, and control against DFU causing bacterial pathogens recorded by *P. odoratissimus* flower extracts which promise it is application as a sustainable traditional medicine against DFU.

Key words: Diabetic foot ulcer, 1,1-diphenyl-2-picrylhydrazyl, invertase inhibition, *Pandanus odoratissimus*

INTRODUCTION

Foot ulcer in diabetic patients is a severe public health issue that results in increased mortality rate among diabetic patients. Globally, the prevalence of diabetic mellitus has increased the occurrence of diabetic-related foot ulceration. Worldwide studies have reported that yearly 9.1 million to 26.1 million diabetic people develop foot ulcers. Diabetic foot ulceration is common in type 2 diabetic patients than with type 1 diabetic mellitus.^[1] This condition may occur due to inappropriate intake of medicine (poor

therapeutic management), not following diabetic diet and improper periodical diagnosis by diabetic patients, and sometimes to chronic diabetic patients which leads to lower limb amputations of leg.^[2]

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The main cause of foot ulcer is due to severe hyperglycemia, poor blood circulation, and nerve damage. These conditions subsequently lead to the formation of Amadori products which further degrade to form several advanced glycation end products (AGEs) precursors and further the formation of AGEs such as pentosidine, N ϵ -carboxymethyl-lysine, and glucosepane. AGEs can create oxidative stress that leads to the necrosis of skin and surrounding tissues.^[3] The hyperglycemic state can also suppress the inflammatory responses and thereby affects the host immune response against the infection. These metabolic imbalances may modify the tissue architecture that becomes susceptible to infections and delayed process of wound healing.

The diabetic foot ulcer (DFU) treatment requires multidisciplinary approach such as management of blood glucose level, wound debridement, and infection management. The enzymes (isomaltase, invertase, heat-stable maltases, lactase, and trehalase) secreted by the mucosal epithelial cells of small intestine plays a major role in the management of hyperglycemia.^[4] These enzymes hydrolyze the disaccharides and increase the absorption of formed monosaccharides into the bloodstream. Inhibition of the above-cited intestinal enzymes can reduce the absorption of several monosaccharides such as glucose, fructose, and galactose, and thereby, it can be considered as an effective strategy in the control of chronic diabetes mellitus.^[5]

Even though there are varieties of antibiotics available in market to control the microbial infections in the foot, drug resistance by the microbes is a challenging task, and hence, an alternative therapeutic approach is necessary to reduce the mortality rate. India is rich in flora and the plants possess numerous medicinal properties such as antidiabetic, antibacterial, antifungal, enzyme inhibitors, antiviral, antimalarial, antioxidant, antihypercholesterolemic, and cardio-, reno-, and hepato-protectiveness property which can act as a better natural based therapeutics to treat various ailments.

Pandanus odoratissimus belongs to Pandanaceae family which has been used widely used in the ayurvedic treatments due to its numerous medicinal properties.^[6] The rationale for the above-cited properties is due to the presence of the various phytochemical/secondary metabolites. The leaf extract possesses components such as carbohydrates, proteins, amino acids, saponins, tannins, phenolic compounds, alkaloids, and flavonoids which may be responsible for the reduction of blood glucose level.^[1] The chloroform extract of leaves is known to promote the wound healing process due to the presence of tannins and flavonoid. The root extract possesses antihyperglycemic properties.^[7] In spite of above-cited reports of *P. odoratissimus*, very minimal scientific documentation is observed for flowers and in that context, our laboratory focused in the investigation and correlation of *in vitro* invertase inhibitory, *in vitro* antioxidant, and *in vitro* antibacterial properties of crude extracts.

MATERIALS AND METHODS

Sample Collection

DFU samples (blood and pus) from patients were collected and provided as sterile cotton swabs (Kind acknowledgment to Dr. N. Thirugnanam, Chief Plastic Surgeon). The collected sterile swabs were immediately inoculated in blood agar plates.

Sample Preparation

Screening of clinical isolates

The collected swab was inoculated on a sterile blood agar plates. The plates were incubated at 37°C for 24–48 h. The colonies obtained were plated on Muller-Hinton agar plates for the single colony isolation by quadrant streaking. Bacterial colonies on the agar plates were subjected for Gram staining method and biochemical tests (urease test and catalase test) for the identification and classification of species.^[8]

Extraction Techniques

Hot water extraction (HWE) method

The *P. odoratissimus* flowers were collected from the local market, Coimbatore, between August and September 2017. The fresh flowers collected were washed with the distilled water and used for the HWE process. Initially, 2 g of finely chopped flowers were subjected to homogenization. Into a clean dry conical flask, added the homogenized flowers and 50 ml distilled water, and boiled in a water bath at 80°C for 5 min. The boiled extract was filtered with Whatman No. 1 filter paper. Into four different aliquots added 20%, 40%, 60%, and 80% of ammonium sulfate and centrifuged at 5000 rpm for 10 min to remove the protein content. The collected supernatant is incubated at 50°C to evaporate the water as a result dried extracts were obtained. These extracts were used for the further experimental analysis.^[9]

Cold percolation extraction (CPE) method

About 3 g of homogenized flowers added 25 ml of the organic solvents (hexane, petroleum ether, chloroform, acetone, methanol, and distilled water) into separate conical flask and kept in an orbital shaker at 110 rpm for 12 h. The extracts were filtered using Whatman No. 1 filter paper. Only the aqueous extract was subjected to ammonium sulfate precipitation with different percentage (20%, 40%, 60%, and 80%) and centrifuged the content at 5000 rpm for 10 min. The collected supernatant was incubated at 50°C to evaporate the water and the resultant supernatant was used for further studies. The other solvent extracts were placed at room temperature for the solvent evaporation, and the resultant supernatant was used for the further experimental analysis.^[9]

***In Vitro* Invertase Inhibitory Assay**

About 1 ml of 40 mM acetate buffered substrate (pH 4.8) is pipetted into the test, control, and blank tubes. To the “control” and “test” tubes, 0.1 ml of invertase enzyme was added. To the “blank” and “test” tubes, 0.2 ml of the flower extract was added and incubated at 37°C for 5 min. Then, 2 ml of DNS reagent was added to all the test tubes and kept in a boiling water bath at 90°C for 7–8 min. Finally, 3 ml of 50 mM sodium acetate buffer was added to all the test tubes. The orange-red color formed was spectrophotometrically measured at 540 nm.^[10] One unit of invertase activity is defined as hydrolysis of 1 μ M of sucrose per minute under assay conditions. Percentage inhibition was calculated by the given below formula:

$$\% \text{ invertase inhibition} = \frac{\text{Enzyme activity of control} - \text{Enzyme activity of test}}{\text{Enzyme activity of control}} \times 100$$

***In Vitro* Antioxidant Assay**

***In vitro* 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Assay**

Free radical scavenging activity of different extracts was tested against the methanolic solution of DPPH. Antioxidants react with DPPH and convert into DPPH. The degree of discoloration indicates the scavenging potential of the antioxidant extract. The change in the absorbance at 517 nm has been used to measure the antioxidant activity. The samples of different extracts were prepared in various concentrations, namely 100, 200, 300, 400, and 500 μ g/ml in methanol. 1 ml of samples with the above-cited concentrations were mixed with equal volume of 0.1 mM methanolic solution of DPPH (0.39 mg in 10 ml methanol). An equal amount of methanol and DPPH was added and used as a control. After incubation for about 20 min under dark conditions, the absorbance was recorded at 517 nm. Experiment was performed in triplicates and the percent scavenging activity was calculated using the following formula:

$$\% \text{ scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

A graph was plotted with concentration (μ g/ml) on X-axis and percentage scavenging on Y-axis and IC_{50} values were calculated, which represents the concentration of the scavenging compound that caused 50% inhibition.^[11]

***In vitro* cupric ion reducing antioxidant capacity (CUPRAC) assay**

In a test tube, 1 ml of $CuCl_2$ solution (1.0×10^{-2} M), 1 ml of neocuproine methanolic solution (7.5×10^{-3} M), and

1 ml ammonium acetate buffer solution (pH 7.0) were added. To this added, 1 ml of extract (sample) to all tubes except blank. The reaction mixture is incubated for 30 min at room temperature and the absorbance was measured spectrophotometrically at 450 nm. A graph was plotted with concentration (μ g/ml) on X-axis and absorbance value on Y-axis, and effective dosage (ED_{50}) values were calculated.^[12]

***In Vitro* Antibacterial Assay**

Agar well diffusion method

Muller-Hilton agar plates were swabbed (sterile cotton swabs) with 16 h old broth culture of respective DFU samples. Wells with 9 mm diameter were bored in each of these plates using sterile cork borer. Different concentrations of plant solvent extracts were added using sterile syringe into the wells and allowed to diffuse at room temperature for 2 h. The plates were incubated at 37°C for 18–24 h and the diameter of the inhibition zone (mm) was measured.^[8]

Broth dilution method

About 100 ml of conical flasks (2 nos.) labeled as “control” and “test” added 50 ml of sterile nutrient broth. The culture was inoculated in both control and the test flasks. 1 mg/ml of the extract was added to the test flask. The test and control were incubated at 37°C in an incubator shaker. The growth of the bacteria is spectrophotometrically measured at 660 nm at different time intervals. A graph was plotted with time (hours) on X-axis and absorbance on Y-axis to study the growth profile of the DFU causing pathogen.

RESULTS AND DISCUSSION

Yield of Phytoconstituents

Extraction is an important step in the screening of phytochemical constituents for the extraction of bioactive constituents from plant materials. Selection of a suitable extraction technique, solvents, and conditions such as temperature, solid: liquid ratio plays an important role for the removal of desirable soluble constituents.^[13] As reported by Singh *et al.*,^[14] extract yield as well as the bioactivities of the extract have been reported to vary in several studies. Higher extraction yield does not necessarily infer that it will have higher antioxidant or antibacterial activity because bioactivity depends on the active compounds present in the extract.

The present studies revealed that the HWE process has provided higher yield than the cold percolation method. The yield obtained for 20%, 40%, 60%, and 80% ammonium sulfate precipitated hot water extracts were 285 mg/g, 607.5 mg/g, 801 mg/g, and 908 mg/g, respectively, and for cold percolation method, the yield was 160 mg/g, 361 mg/g, 558 mg/g, and 759.3 mg/g, respectively.

In vitro Invertase Inhibition

In the present study, the methanolic extract of *P. odoratissimus* flowers was found to have an effective *in vitro* invertase inhibitory activity ($62.5 \pm 3\%$). The result of Dixon plot has revealed a mixed type of inhibition produced by the inhibitor compound present in the methanol extract. The inhibitor constant (K_i) was found as $400 \mu\text{g}$ for 10 mM substrate concentration and $560 \mu\text{g}$ for 20 mM substrate concentration, and hence, $400 \mu\text{g}$ of inhibitor was considered as an effective concentration in controlling at least 50% invertase inhibition [Figure 1].

According to studies documented by Sathishkumar *et al.*,^[9] *in vitro* invertase inhibitory activity was observed in seeds of *Manilkara zapota* (98.7%) and *Artocarpus heterophyllus* (35.6%), and the activity was due to the presence of polyphenols. It was reported that invertase inhibitors interrupt the digestion and adsorption mechanism of sucrose, and thus hindering postprandial hyperglycemia. Similarly, the presence of secondary metabolites in *P. odoratissimus* flowers may play a significant role in managing diabetes mellitus by inhibiting invertase enzyme.

In vitro Antioxidant Assay

In vitro CUPRAC assay

The chromogenic redox reagent used for the CUPRAC assay was bis (neocuproine) copper (II) chelate. The Cu(I)-neocuproine (Nc) chelate was formed as a result of the redox reaction of chain-breaking antioxidants present in the test sample with the CUPRAC reagent which yields an yellow color Cu(I)-Nc complex and an increase in the color revealed the concentration of antioxidants.^[12] The results revealed that crude methanolic and the aqueous extracts have potential antioxidant activity. As of now, there are no reports on the cupric ion reducing potential of the *P. odoratissimus* flowers, and our laboratory is first to record the above-cited activity. The ED_{50} of methanolic and aqueous extracts was found to be $455.3 \pm 8 \mu\text{g/ml}$ and $452.14 \pm 5 \mu\text{g/ml}$, respectively [Figure 2].

In vitro DPPH Assay

From the results obtained, the methanolic extract (CPE) and the ammonium sulfate fractionate of aqueous extract (HWE) possessed highest antioxidant activity. The investigation also revealed that the chloroform and acetone extracts possessed moderate antioxidant activity while the hexane extract has recorded poor activity. The IC_{50} of CPE and HWE was found to similar, i.e. $260.6 \mu\text{g/ml}$, and the CPE has recorded a strong dipricryl radical scavenging activity ($90.7 \pm 2\%$) than HWE ($81.4 \pm 3\%$). The highest activity of CPE was due to the presence of polyphenols and flavonoid presents in the extract.^[15]

In Vitro Antibacterial Assay

Agar well diffusion assay

The acetone extract exhibited significant antibacterial activity against *Escherichia coli* followed by methanolic extract and chloroform extract. The zone of inhibition was measured and the data have been shown in Table 1. Similarly, a positive control (streptomycin) results were also depicted in Table 2. As per the

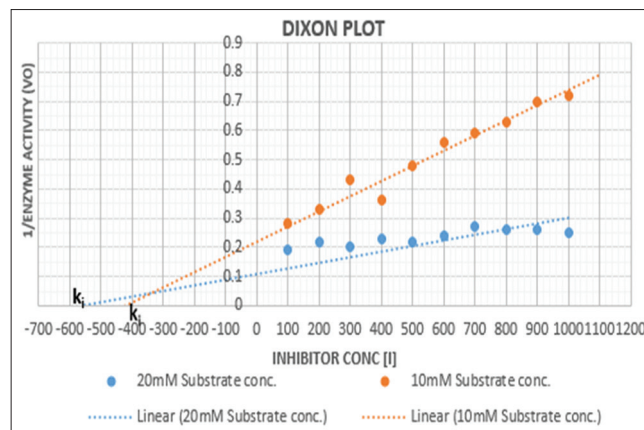


Figure 1: Dixon plot of methanolic extract of *Pandanus odoratissimus*

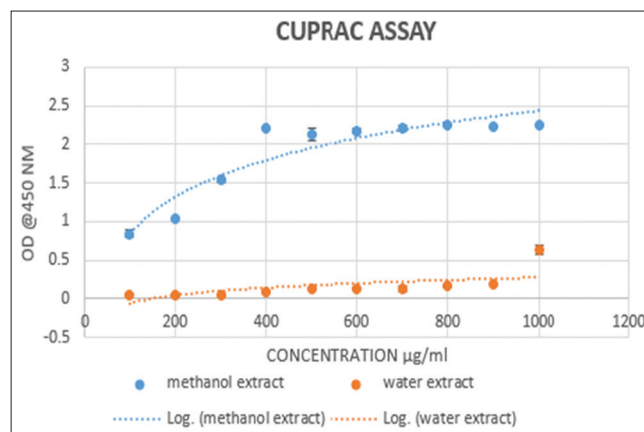


Figure 2: Cupric ion reducing antioxidant capacity of methanolic and hot water extraction of *Pandanus odoratissimus*

Table 1: Antibacterial activity of *P. odoratissimus* flowers against *E. coli* isolated from DFU affected patients

Extracts	Zone of inhibition (mm)	
	Concentration ($\mu\text{g/ml}$)	
	50	100
Acetone	10 ± 2	16 ± 4
Chloroform	6 ± 3	10 ± 2
Aqueous	No inhibition	No inhibition
Methanol	10 ± 4	12 ± 3

P. odoratissimus: *Pandanus odoratissimus*, *E. coli*: *Escherichia coli*, DFU: Diabetic foot ulcer

report by Kumar *et al.*,^[16] the alcoholic extract of *P. odoratissimus* leaves exhibited effective zone of inhibition against *E. coli*. The leaves of *P. odoratissimus* possess antibacterial activity only against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. However, the present investigation of flower extracts has shown effective inhibition against the Gram-negative bacteria *E. coli* (acetone (16 ± 4 mm) and methanolic extracts (12 ± 3 mm)) and poor inhibition against *S. aureus* and *Pseudomonas aeruginosa* at 100 µg/ml concentration. Aqueous extract does not show any inhibition against the isolated species.

Growth pattern of the pathogenic bacteria

The growth inhibitory pattern of *S. aureus* revealed that 50% of growth was controlled at the 2nd h and 30.64% inhibition was recorded at the 5th h. For *E. coli* at the 2nd h, a moderate inhibition of 22.4% was observed for the crude extract. In our investigation, the growth pattern of *P. aeruginosa* revealed that the presence of crude extract has moderate inhibition of 29.58% at 24th hr and inhibition was declined to 14% after 24 h. Although inhibition percentage was low compared to other pathogens, the crude extract was able to control almost 30% of the growth until 24th h [Figure 3].

Table 2: *In vitro* antibacterial activity of streptomycin (positive control) against bacterial pathogens isolated from DFU affected patients

Bacterial species	Zone of inhibition (mm)	
	Concentration of streptomycin (µg/ml)	
	50	100
<i>E. coli</i>	15±3	20±4
<i>S. aureus</i>	13±2	18±3
Unknown species	11±3	17±4
<i>P. aeruginosa</i>	30±3	35±2

E. coli: *Escherichia coli*, DFU: Diabetic foot ulcer, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. aureus*: *Staphylococcus aureus*

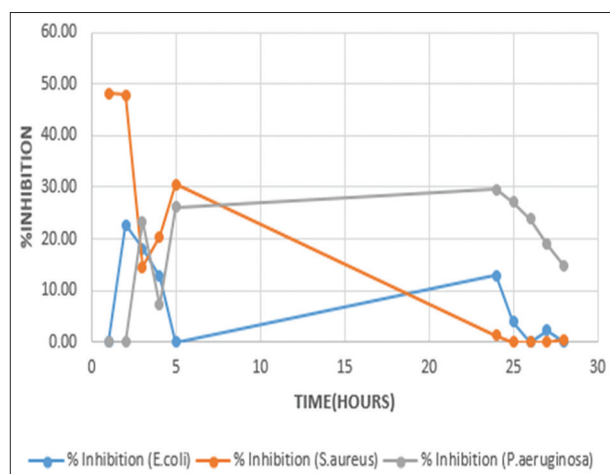


Figure 3: Growth inhibitory pattern of diabetic foot ulcer-causing pathogens

The crude extract has moderate inhibition pattern against the *E. coli* and *S. aureus*. However, the previous documentation by Faras *et al.*^[17] on the antibacterial activity of the *Pandanus amaryllifolius* leaves revealed that the water and ethanol extract stimulates the growth of *E. coli* and *S. aureus* due to the reducing sugar content in the leaves.

CONCLUSION

The current studies proved that the *P. odoratissimus* flowers extract has strong antioxidant property. The selected plant extract has significant *in vitro* invertase inhibitory activity. Thus, the antioxidant potential and the invertase inhibitory property exhibited by the plant extract can control the chronic diabetes mellitus. There was satisfactory antibacterial activity observed in the crude extract against *E. coli* and *S. aureus* isolated from DFU affected patients. The studies proved that *P. odoratissimus* flowers extract can serve as a potent herbal medicine to treat DFU. Further studies are required to find the causative phytochemicals for the above-cited properties.

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