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Article

Purified novel and new diferuloyl glycerate related phenolic acid from *Pandanus odoratissimus* flowers shows antioxidant, invertase inhibition and control against diabetic foot ulcer (DFU) causing bacterial pathogens – *An in vitro* study to establish an effective regulation over type 2 diabetes mellitus

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Chronic type 2 diabetes mellitus (T2DM) and its associated diseases are major concern among human population and also responsible for significant mortality rate. Hence, the present study aims to evaluate and correlate the invertase inhibition, antioxidant activity and control against DFU causing bacterial pathogens by *Pandanus odoratissimus* flowers. Two dimensional preparative thin layer chromatography (2D PTLC) was adopted to purify the phenolic acid component and LC-MS^2 was done to predict the phenolic acid structures. Standard spectrophotometry methods were adopted to investigate the *in vitro* invertase inhibitory and antioxidant (CUPRAC and ABTS) activities. Agar well diffusion and broth dilution assays were used to record the antibacterial property against DFU causing pathogens isolated from clinical samples. Statistical analyses were used to validate the experiments. A new and novel diferuloyl glycerate related phenolic acid (*m/z* 442) purified from PTLC eluate has recorded satisfactory cupric ion reducing power (ED_{50}= 441.4±2.5 µg), moderate ABTS radical scavenging activity (IC_{50}= 450.3±10 µg; 32.5±1.5%), and a near moderate, *in vitro*, invertase mixed type inhibition (24.5±4.5%; K_i: 400 µg). Similarly, bacterial growth inhibitory kinetics has showed a significant inhibition against *E. coli* and *S. aureus.*

Keywords: ABTS. CUPRAC. Diabetic foot ulcer. Diferuloyl glycerate. Invertase inhibition. *Pandanus odoratissimus.*

INTRODUCTION

Type 2 Diabetes mellitus (T2DM) is a chronic metabolic disease that has affected more than 415 million people globally and is expected to rise to 642 million by the year 2040 (IDF Diabetes Atlas, 2015). India stands rank two with approximately 69 million people affected by T2DM. The predicted reasons for T2DM is decreased intake of fibre, low physical activity, increased intake of refined cereals and mental stress which leads to long term complications such as coronary artery disease, diabetic neuropathy, nephropathy, and retinopathy, and diabetic foot ulcer (Unnikrishnan *et al.,* 2016). Among various complications, diabetic foot ulcer (DFU) is a major concern and about 25% human population is affected by DFU. The pathophysiology of DFU includes high body mass index, increased level of glycated hemoglobin (HBA1c), foot deformity, high

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plantar pressure and microbial infections (Bortoletto *et al.,* 2014). DFU normally leads to disrespect in society, imbalance in finance, mental stress, and can be prevented carefully through appropriate awareness and extreme care in maintenance of foot (Pendsey, 1994). Hence, an effective maintenance of glycemic index is important to control acute and chronic diabetes mellitus..

India is rich in flora and many plants have been scientifically documented for their numerous medicinal properties. Even though, plants have been reported for their antidiabetic properties through traditional practice, only minimal scientific evaluation has been documented in literature. One such plant is *Pandanus odoratissimus,* belonging to Pandanaceae family that is distributed worldwide and in India it is widespread in the coastal areas. The leaves, flowers and root extracts are traditionally used to treat various ailments such as syphilis, scabies, small pox, leprosy, tumors and leucoderma, and possess carbohydrates, proteins, saponins, tannins, sterols, terpenes, alkaloids, phenolics, flavonoid, α-terpineol, β-carotene, β-sitosterol andviridine (Sanjeeva *et al.,* 2012; Adkar, Bhaskar, 2014). The fruit and root extracts has recorded the antioxidant, antibacterial, antihyperglycemic and cytotoxic properties (Venkatesh *et al.,* 2012; Andriani *et al.,* 2015). Previous report by Suvetha *et al.,* (2018) has proved significant cupric ion reducing power, invertase inhibition and DFU causing bacterial pathogen inhibitory effects of aqueous and methanolic flower extracts of *Pandanus odoratissimus*.

In Lieu of above cited reports, there is no scientific documentation about the antioxidant, invertase inhibitory and control against DFU causing bacterial pathogens of purified phenolics from flowers. So, the present study has focused to investigate the above cited properties through standard parameters.

MATERIAL AND METHODS

Chemicals

Yeast invertase (M.P. Biomedicals), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid [ABTS] (Sigma Aldrich), Neocuproine, copper (II) chloride and β-cyclodextrin (Merck), and Muller Hilton agar (HiMedia). All other chemicals and solvents used in the experimental analysis were of analytical grade.

Collection and preparation of methanolic flower extracts using cold percolation extraction (CPE) method

The flowers were collected from the local market (Procured from farmers), Coimbatore, Tamil Nadu, India between Aug' – Sep' 2017, and the voucher specimen was deposited in the Department of Biotechnology, Kumaraguru College of Technology, Coimbatore (No. DBT/KCT/Aug/23/2017-18/PO-01). The fresh flowers were washed with the distilled water and used for CPE process (Suvetha *et al.,* 2018).

One dimensional thin layer chromatography (1D TLC) analysis

Silica gel G dissolved in water was applied as thin layer (0.25 mm) to the glass plate (20 cm × 10 cm) with the help of an applicator. The plates were activated at 100°C for 30 minutes in a hot air oven. About 25 µl of the methanolic extract was spotted and the chromatogram was developed one dimensionally in an air-tight chamber in the presence of mobile phase consists of ethyl acetate - ethanol - water (5:1:5). The plates were then visualized under far UV light (360 nm) to detect the phenolics present in extracts (Sathishkumar *et al*., 2015).

Two dimensional preparative thin layer chromatography (2D PTLC) analysis

The procedure adopted for 1D TLC was followed for preparative thin layer chromatography (PTLC) with the exception of thickness of silica gel (2 mm) and the volume of the test sample (500µl). The Two dimensional mobile phase was as follows:

First dimension: Ethyl acetate - ethanol - water (5:1:5)
Second dimension: Acetone: acetic acid (17:3)

After the development of chromatogram, the visualized strong spots were carefully eluted and suspended in phosphate buffer (pH 7.0), and centrifuged at 10,000 rpm for 10 minutes. The resultant supernatant was lyophilized and subjected for experimental analysis (Sathishkumar *et al.*, 2015).

Digital image processing of TLC and PTLC plates using OTSU method

To enhance S/N ratio of TLC plate, image processing by an improved threshold technique (Otsu method) was adopted as per the protocol described by Reddy *et al.,* (2017).

High performance liquid chromatography – mass spectrometry (HPLC-PDA-ESI/MS_2) analysis

The liquid chromatography electron spray mass spectrometry (LC-MS) analysis was performed according to the method described by Sathishkumar *et al.,* (2013). The obtained base peak was further subjected to MS^2 scan (0.062 min; scan range: 131-452) to identify the phenolic compound.

Cupric ion reducing power antioxidant (CUPRAC) assay

The protocol proposed by Özyürek *et al.,* (2011) was used to investigate the cupric ion reducing property. A graph was plotted with concentration (µg/ml) on X-axis and absorbance value on Y- axis and ED_{50} value were calculated. Ferulic acid was used as standard for the construction of calibration curve.

In vitro ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay

A slightly modified method of Re *et al.,* (1999) was adopted for ABTS assay. Ferulic acid was used as standard for the construction of calibration curve. Percentage inhibition was calculated by the given below formula:

% ABTS radical scavenging = (1 – [Absorbance of test/ absorbance of control]) × 100.

In vitro invertase inhibitory assay

The method described by Melius (1971) was used to study the invertase inhibitory activity. One unit of invertase activity is defined as the hydrolysis of 1 µM of sucrose per minute under assay conditions. Percentage inhibition was calculated by the given below formula:

% invertase inhibition = (1 – [Enzyme activity of test / Enzyme activity of control]) × 100.

Sample Collection from DFU affected patients and screening of bacterial pathogens

About twenty five samples (blood and pus of DFU affected patients) were collected under the supervision of Dr. N. Thirugnanam, Chief plastic surgeon, and were provided as sterile cotton swabs**.** The sterile swabs were immediately inoculated in blood agar plates and incubated at 37°C for 24 to 48 hrs. The obtained colonies were subjected for quadrant streaking in Muller Hinton agar plates for isolation of single colony. The obtained pure colonies were subjected for Gram staining and biochemical tests (urease test and catalase test) for the identification and classification of species (Species were confirmed under the guidance of Dr. N. Thirugnanam).

Agar well diffusion and broth dilution method

The pure colonies were inoculated in Muller Hinton agar (agar well diffusion), and nutrient agar (broth dilution), respectively, and the subsequent steps were followed as per the procedure proposed by Arumugam *et al.,* (2012). Streptomycin was used as a positive control.

Investigation of drug release kinetics (Kneading method) from a fabricated bioimplant

The drug release kinetics (*in vitro* invertase propery and ABTS assay) was investigated according to the method espoused by Ghosh *et al.,* (2011). The physiological condition was simulated in terms of 0.02 M phosphate buffer (pH 8) at 37ºC and the bioimplant was introduced

into the system. The release profile was continually monitored from 0 to 3 hrs with 30 minutes time interval and studied as per Hixson – Crowell model (Divya *et al.,* 2017).

Statistical analysis

The values depicted in the results were expressed as mean ± s.d. Karl Pearson correlation coefficient, ED_{50} and IC_{50} values were calculated using MS excel 2010 version. Dixon plot was drawn using MS excel 2010 version. MATLAB 15a was used for the digital image processing studies.

RESULTS AND DISCUSSION

In recent years, in order to increase the resolving capacity of the TLC technique, a two dimensional mode of purification has been widely exercised by the scientific community. In this method, one stationary phase and two different mobile phases was adopted to have better results for the purification of complex matrix material (Rabel, Sherma, 2016). Hence, 2D PTLC has been adopted for purification of polyphenols.

In the present investigation, 1D TLC analysis of methanolic extract of *Pandanus odoratissimus* has revealed a single blue color spot (phenolic acid fraction) with R_f value of 0.64 under far UV light **(Figure 1A)**. Previous reports of TLC and HPLC analyses in root and leaf extracts have perfectly matched with our current result (Sasikumar *et al.,* 2009; Ghasemzadeh, Jaafar, 2013). The identified polyphenols were purified using 2D PTLC technique and the results have clearly proved an improved resolving power, where, under one dimension version only a single blue color spot (phenolic acid) **(Figure 1B)** and in two dimension, two spots (One light yellow color weak spot [flavonoid] and one blue color strong spot [phenolic acid]) were observed **(Figure 1C)**. The issue in the elution of polyphenols using planar chromatography is to optimize the signal/ noise (S/N) ratio in the TLC plate for identification of exact distribution area of polyphenols (e.g., overlapping of object color [blue/dull yellow etc.] with background color [ultraviolet]). An early attempt made by Yu *et al.,* (2016) has demonstrated the overcoming of shot and thermal noises (CMOS sensors) through different image segmentation algorithms to process the TLC plate images. In our current study, OTSU based image processing algorithm was used (Reddy *et al.,* 2017) and the threshold level at 80 was found to be the optimal in identifying the exact distribution area of phenolics **(Figure 2A & B)**. Finally, the identified strong blue color spot was eluted and subjected for LC MS^2 analysis.

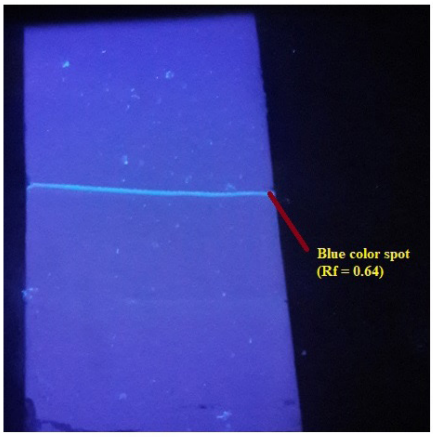

FIGURE 1A

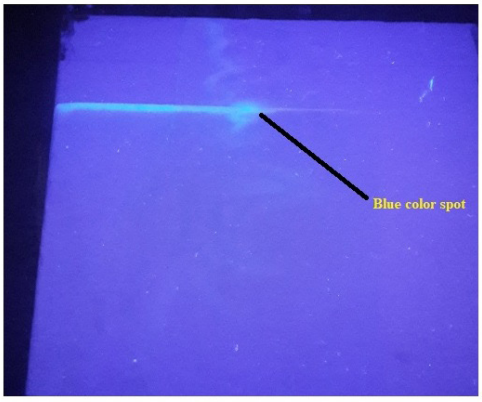

FIGURE 1B

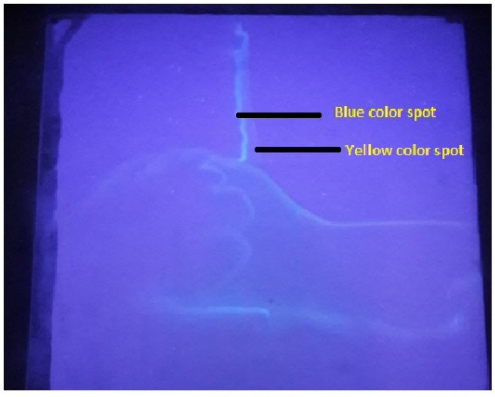

FIGURE 1C

FIGURE 1 -(A): One dimensional thin layer chromatogram of methanolic extract of *Pandanus odoratissimus* developed and visualized under far UV light; Preparative thin layer chromatogram (PTLC) developed and visualized under far UV light **(B):** One dimensional view; **(C):** Two dimensional view.

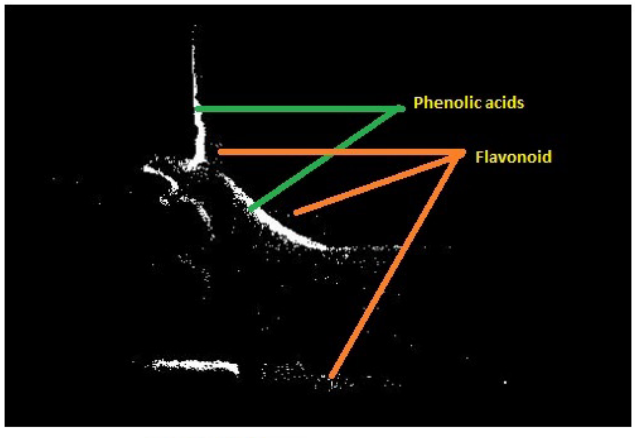

FIGURE 2A

FIGURE 2B

FIGURE 2 - Digital image segmentation (OTSU method) analysis of PTLC plates: **(A):** Image segmented view (Gray scale); **(B)**: Original PTLC plate view.

The HPLC result proved the presence of a single compound at the retention time (R_t) of 8.47 min **(Figure 3A)**, andfurther recorded MS analysis at *m/z* 442 (100%), and MS^2 analysis at *m/z* 337 (100%), *m/z* 425 (14%), 235 (48%) and 193 (20%), respectively has confirmed the presence of novel diferuloyl glycerate related phenolic acid. The peaks at *m/z* 425 revealed the loss of hydroxyl group (-OH) [-17 amu loss], *m/z* 337 demonstrated the cleavage of C=C bond with the loss of phenolic ring derivative [-105 amu loss], *m/z* 235 recorded the loss of protonated ferulate [-207 amu loss], and *m/z* 193 revealed the loss of feruloyl glycerate related moiety which left the residue as ferulic acid **(Figure 3B)**. Reports by (Kang *et al.,* 2016) [MS^2 [443]: 235(100), 207(60), 193(30)] and (Jiménez-Sánchez *et al.,* 2016)[MS^2 [443]: 193 (100), 249(15)] on the fragmentation pattern of ferulic acid derivative has supported our results, and confirmed the presence of a new and novel diferuloyl glycerate related phenolic acid in *Pandanus odoratissimus* flowers.

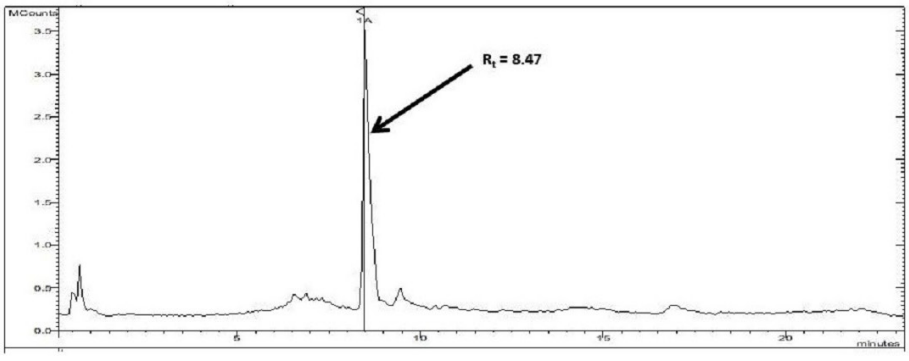

FIGURE 3A

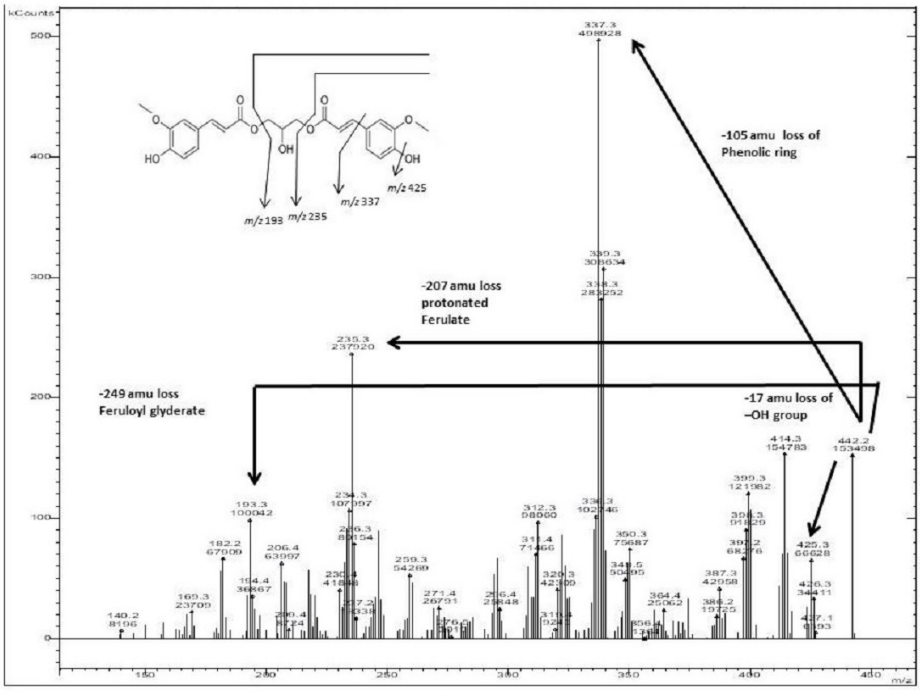

FIGURE 3B

FIGURE 3 - (A): HPLC analysis of PTLC eluate; **(B):** MS^2 analysis and fragmentation pattern of PTLC eluate.

The present study on the investigation of antioxidant property of purified diferuloyl glycerate related phenolic acid has recorded a significant dose dependent cupric ion reducing power (R^2 = 0.85; ED_{50}= 441.4 ±2.5µg)compared with ferulic acid (R^2= 0.99;ED_{50}= 459.43 µg). Previous studies in the root and leaf extracts have adopted FRAP method to investigate the reducing power capacity (Sasikumar *et al.,* 2009; Ghasemzadeh, Jaafar, 2013), whereas, we selected CUPRAC because of its faster kinetics and the ability of Cu(II) ion to oxidize both lyophilic and hydrophilic antioxidants (Apak *et al.,* 2013). Earlier our laboratory has established the significant cupric ion reducing power of aqueous and methanolic flower extracts (Suvetha *et al.,* 2018). Similarly, radical scavenging activity was measured using ABTS assay because of its ability to react with antioxidants in a wide spectrum pH, solubility in both organic and aqueous solvents, and faster steady state reaction kinetics (Shalaby, Shanab, 2013). The investigation has revealed a moderate radical scavenging activity of diferuloyl glycerate related phenolic acid (R^2 = 0.99; IC_{50} = 450.3 ±10µg; 32.5±1.5%) compared with ferulic acid (R^2 = 0.98; IC_{50} = 454.86 µg; 90.5±0.7), and a report documented by Compton *et al.,* (2012) on 1,3-diferuloyl glycerol's scavenging activity has supported our result. The antioxidant mechanism of diferuloyl glycerate related phenolic acid is mainly due to the presence of phenolic hydroxyl groups (-OH). They can interact with unstable free radicals through the donation of a hydrogen radical, thus forming a very stable phenoxyl radical. The unpaired electron present in the phenoxyl radical is displaced to various positions across the structure and an extended methoxy radical side chain could be the probable reason for its great stability. Likewise, the carboxylate ion present in the phenolic ring acts as free radical attack site and prevent the cell membrane from oxidation (Srinivasan *et al.,* 2007; de Oliveira Silva, Batista, 2017).

The purified diferuloyl glycerate related phenolic acid has showed a satisfactory invertase inhibitory effect (K_i= 400 µg) and recorded a mixed type of inhibition (24.5±4.5%) **(Figure 4)**. Earlier report has established a near satisfactory invertase inhibitory property of methanolic flower extract (Suvetha *et al.,* 2018). Inhibition of intestinal carbohydrases is a classic therapeutic approach to control chronic diabetes mellitus, and previous scientific records has proved a satisfactory invertase inhibitory effect of standard phenolic acid and its derivatives, and the inhibitory mode was found to be mixed type (Adisakwattana *et al.,* 2009; Kalita *et al.,* 2018). The exact mechanism of interaction of phenolic compounds with invertase is still unknown, but, it is expected that the hydroxyl, carbonyl, and aromatic groups present in the diferuloyl glycerate related phenolic acid may interfere with the interactions between catalytic site amino acids and sucrose which, prevents the accessibility of sucrose across the site (Sainz-Polo et al., 2013). Hence, the above cited process leads to deterrence of glucose absorption across the intestinal villi, and thereby, an effective control over glycemic index in blood.

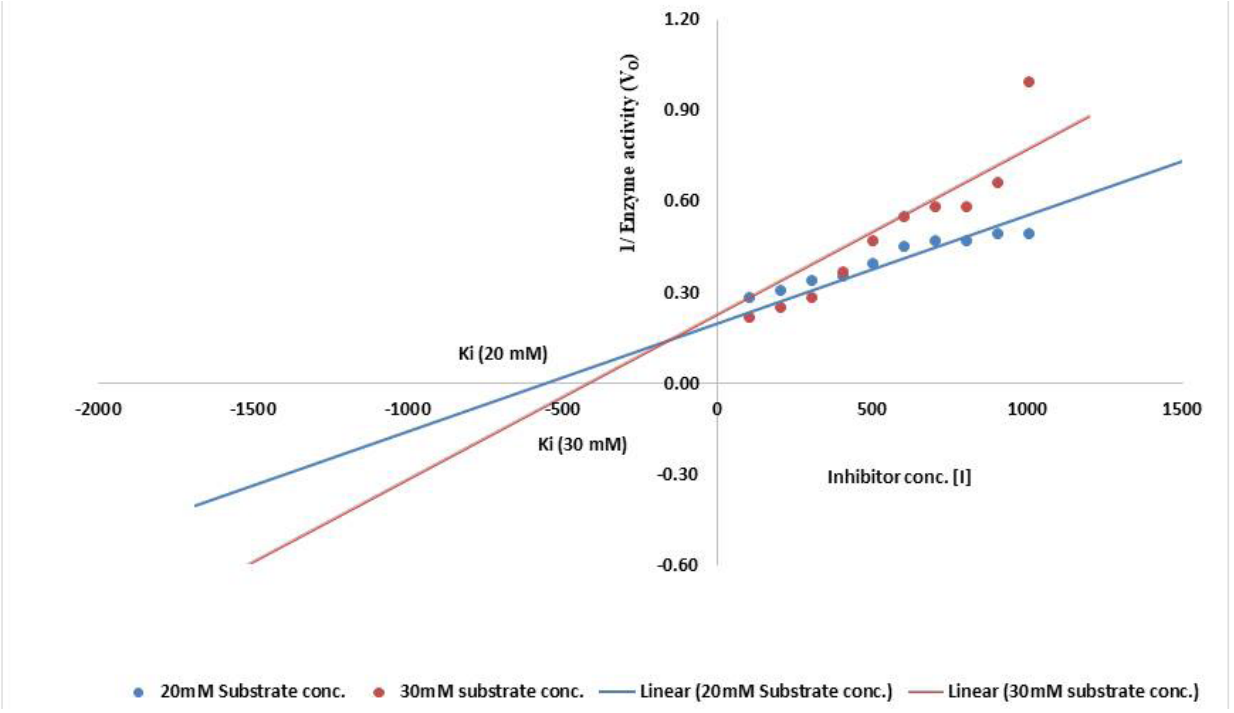

FIGURE 4 - Mixed type of invertase inhibition (Dixon plot) by diferuloyl glycerate related phenolic acid

Current investigation has revealed the predominance of *E. coli* (40% population), *S. aureus* (40% population) and *P. aeruginosa* (20% population) in DFU affected clinical samples. The bacterial growth inhibitory kinetics (broth dilution method) of diferuloyl glycerate related phenolic acid (PTLC eluate) has showed a significant inhibition against *E. coli* (94.5±2.5%) and *S. aureus* (92.9±1.8%), and poor inhibition against *P. aeruginosa* (18.1±2.6%). Maximum inhibition of all the species were observed between 3rd – 5th hour. But, the well diffusion assay results showed a moderate inhibitory property of diferuloyl glycerate related phenolic acid (100 µg) against *E. coli* (14 ± 2) and *S. aureus* (13 ± 1), and poor inhibition against *P. aeruginosa* (7± 2) (**Table I**). Previous study on the fruit extracts of *P. tectorius* has shown a near moderate inhibition against*E. coli*, *S. aureus* and *P. aeruginosa,* and the growth inhibitory time has been recorded > 8 hrs (Andriani *et al.,* 2015). In the current study, a similar report was recorded for zone inhibition, but, an effective inhibitory kinetics (i.e., 5 hrs ≤) was noticed for the purified phenolic molecule. Similarly, an early report has recorded very poor inhibitory property of flower extract against *E. coli* and *S. aureus* (Suvetha *et al.,* 2018) which proved the efficacy of purified diferuloyl glycerate related phenolic acid in controlling DFU causing pathogens. Likewise, the previous results recorded for standard ferulic acid against *E. coli* (9.25 ± 0.25), *S. aureus* (9.25 ± 0.25)and *P. aeruginosa* (9.0 ± 0) was near moderate (Saavedra *et al.,* 2010), and our current study has proved superiority of purified diferuloyl glycerate related phenolic acid than ferulic acid.

The present investigation has proved a regulated release of diferuloyl glycerate related phenolic acid which is monitored through invertase inhibition and ABTS radical scavenging activities. The invertase inhibition of diferuloyl glycerate related phenolic acid coated in a bioimplant has recorded 16.83±1.1% after 60 minutes, and till 90 minutes, only 18.75±2.1% inhibition was observed which proved a consistent and slow release of phenolic acid. This slow release profile continued and after three hours a maximum inhibitory activity of about 43.09±3.4% was recorded. The result is contradictory (i.e.,1.75 fold high inhibition) when compared to the inhibitory activity (24.5±4.5%) of free diferuloyl glycerate related phenolic acid which proved the effectiveness of the immobilized phenolic acid coated on the bioimplant. The release

kinetics as per Hixson – Crowell model has established a strong correlation co-efficient ($R^2 = 0.89$) and k-value (2.55). The radical scavenging activity of bioimplant coated diferuloyl glycerate related phenolic acid at initial time period (after 30 minutes) was 6.95±0.6% and the release rate of the drug was highly controlled even after three hours (15.46±2.7%). A strong correlation co-efficient ($R^2 = 0.97$) and k-value (1.15) was recorded between the drug release and its scavenging property. So far, no reports were available of bioimplant coated phenolic acid release and our laboratory was first to document the above cited research finding.

TABLE I - Antibacterial activity of purified diferuloyl glycerate related phenolic acid and streptomycin (positive control) against DFU causing pathogens (clinical isolates)

Bacterial pathogens	Zone of inhibition (mm)			
	Concentration of streptomycin (µg/ml)		Concentration of diferuloyl glycerate related phenolic acid (PTLC eluate) (µg/ml)	
	50	100	50	100
E. coli	15 ± 3	20 ± 4	9 ± 2.5	14 ± 2
S. aureus	13 ± 2	18 ± 3	7 ± 2	13 ± 1
P. aeruginosa	30 ± 3	35± 2	6 ± 3	7± 2

CONCLUSION

A novel and new diferuloyl glycerate related phenolic acid was purified from *Pandanus odoratissimus*flowers. The purified phenolic acid has proved a promising reducing and radical scavenging properties, significant invertase inhibition and effective control over DFU causing pathogens. Further studies can be extended in experimental animals to find the efficacy/ dosage of the drug and its role in modern medicine.

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