Clinical Phytoscience

ORIGINAL CONTRIBUTION Open Access

Exploration of the role of a lithophytic fern, *Pteris vittata* L. in wound tissue regeneration and remodelling of genes in hyperglycaemic rat model

Tania S. Paul[1*], Biswadeep B. Das[2], Yogesh P. Talekar[2] and Suchitra Banerjee[3]

Abstract

Background: In hyperglycemic conditions like diabetes, impaired wound healing occurs due to endothelial damage, dysfunction of leukocyte, decreased phagocytosis and secondary infection which may lead to amputation and debility. Ethnomedicinally, *Pteris vittata* L. (PV) is used for wound healing. This fern is arsenic hyper-accumulator but its therapeutic aspect is still unexplored. Hence, the present study was put forth to study its aqueous extract and ethanolic extract in diabetic wound healing.

Methods: Rats were divided into diabetic control, povidine iodine (PI) treated, ethanolic and aqueous extracts of PV treated groups ($n = 6$). Circular excision wound closure was observed for 15 days with and without treatment. After study completion, skin was divided into four sections wherein first section was homogenized for collagen, hydroxyproline and hexosamine assay. Second, third and fourth sections were used for antioxidant assay, gene expression and histopathology. Column purified fraction of ethanolic extract of PV was subjected to High Performance Liquid Chromatography, Fourier-transform infrared spectroscopy, Nuclear Magnetic Resonance and Mass spectroscopy. Data obtained were analyzed using one way analysis of variance and expressed as Mean ± SD.

Results: The percentage difference in wound area of day 15 to day 0 showed 65% wound contraction in diabetic control rats. The percentage reduction in wound area showed by PI and extracts of PV were 79% and 85% respectively. Statistical significant increase in collagen, hydroxyproline and hexosamine was observed in the test groups as compared to disease control and PI treated rats. Similarly, statistical significant increases in antioxidant enzymes were observed in the treated groups with decrease in lipid peroxidation. Treatment of rats with PI and two extracts of PV up-regulated Matrix Metalloprotein-9, Collagenase-2 and VEGF-1 and down regulated Tumor Necrosis Factor- α and Interleukin-6. Histopathology in diabetic rats showed incomplete scab formation with haemorrhages which were absent in treated rats. Spectral data showed presence of polyphenolic compounds, fatty acids and ascorbic acid.

Conclusion: Alternative and complimentary management based on herbal biotherapy which can promote angiogenesis, increase collagen and lower the levels of reactive oxygen species are warranted for healing of wounds in hyperglycaemic conditions which were achieved by two extracts of PV.

Keywords: *Pteris vittata*, Hyperglycemia, Excision wound, Tissue antioxidant, Tissue biochemistry, Gene expression, Histopathology

* Correspondence: taniapaul6@gmail.com
[1]APT Research Foundation, DSIR-SIRO Recognized Research Institute, Sinhagad Road, VadgaonKhurd, Pune, Maharashtra 411041, India
Full list of author information is available at the end of the article

Introduction

Impaired wound healing in hyperglycemic conditions is a complicated syndrome as it is a major cause of amputations. Although adequate and appropriate care is given to manage diabetic wounds it persists for longer time [1]. It is postulated that the main etiology of this disease may be elevated blood glucose levels causing endothelial damage with consequent occlusion of capillary vessels, dysfunction of leukocyte and decreased phagocytosis resulting in impaired wound healing leading to risk of infection [2].

International Diabetes Federation reports that 9.1–26.1 million people develop diabetic foot ulcers (DFU) annually and development of a DFU is associated with 5% mortality in the first 12 months and 42% mortality within 5 years [3]. Recently it was reported that the estimated global cost of diabetes in 2015 was $1.3 trillion and it is believed that up to one-third of diabetes expenditure is on lower-limb–related problems [4]. Major interventions used nowadays for management of wounds in diabetics include debridement, irrigation, antibiotics, tissue grafts and proteolytic enzymes [5]. Advanced therapies including growth factors, extracellular matrices (ECMs), engineered skin, and negative pressure wound therapy [6]. In diabetic condition, an injury can lead to large recruitment of neutrophils and macrophages in the wound bed which creates influx of pro-inflammatory cytokines (such as IL-1β, IL-6, and TNF-α) and reactive oxygen species (ROS). ROS in small quantity is quintessential but excessive production or its impaired detoxification may cause non-healing chronic wounds by inhibiting proliferation of fibroblasts and keratinocytes [7]. Fibroblasts are gathered to form granulation tissue, which lay down collagen, after getting stimulation from macrophages that eventually results in the formation of a scar. During remodeling of wound, matrix metalloproteinases (MMPs) play a major role which cleaves collagen. However, glycated collagen is also more resistant to MMP-mediated degradation that disrupts matrix modeling and delay in diabetic wound healing [8].

Therefore agents that can increase angiogenesis, increase collagen and lower the levels of ROS are warranted for effective healing of wounds in hyperglycaemic conditions.

Pteridophytes are medicinally important plants that were dominant plant group 250 million years ago. In India the maximum number of pteridophytes occurs in the Eastern Himalayas and about 400 species of pteridophytes are found in Western Ghats and Southern India [9]. Among these pteridophytes, PV is a lithophytic fern, which is commonly known as 'brake fern' [10]. This Chinese brake fern is native to China but it now widely naturalized in many areas of the world including Southern Africa and Tropical Asia [11]. PV mostly grows along roadsides and on almost any calcareous substrate such as old masonry, sidewalks, limestone habitats, building crevices, and in habitats with alkaline pH, such as sites contaminated with arsenic [12]. In light of its extreme tolerance for arsenic, PV has been the subject of intense examination since its discovery as an arsenic hyper accumulator. The plant is reported to have flavonoid rutin along with substantial amounts of phenols that elicits antimicrobial and astringent property [13], antitumor, platelet aggregation and anti-inflammatory action [14]. This fern is also reported to possess hypotensive as well as antiviral activity [15]. PV generally contain rutin, kaempferol monoglycoside, kaempferol diglycoside, quercetin monoglycoside, and quercetin diglycoside [16]. Another species of Pteridaceae family, *Pteris tripartite* was studied for its chemical constituent and in its ethanol extract an octadecanoic acid (a stearic acid) was found to be present [17]. The methanolic extract of PV showed suppression of SOS response in Escherichia coli PQ37, which is a promising strategy for tackling antibiotic resistance in *E.coli* and also proves its antimutagenic effect. It also showed antioxidant potential and anti-proliferative action on human MCF-7 breast cancer cells [18].

Different species of the genus Pteris have different ethnomedicinal values for example, the species *Pteris biaurita* L. is used in Similipal Biosphere Reserve, Orissa, India for treating wounds, cuts and bruises [19]. Ethnomedicinally, the whole plant of PV is made into paste and used for healing of wounds by applying externally in Tamil Nadu, India [20]. In Uttarpradesh, India, leaf juice of PV is ethnomedicinally used to relieve blisters on the tongue [21]. A recent study to explore the possible effects of PV on visual sensitivity and electroretinography (ERG) waves was done and the results indicated that the extract significantly augmented dark- and light-adapted ERG b-wave amplitude and improved the visual sensitivity by 0.8 log unit of light intensity, and reduced the regeneration time for rhodopsin [22]. Nowadays it is believed that PV cultivation should be promoted as a medicinal plant due to its rich phenolic and flavonoid content and can be used for preparing health care products for age related chronic diseases [23]. Our previous study on PV showed its hypoglycaemic property in diabetic rats [24], thus, in conjunction to our previous experiment and its ethnomedicinal claims on wound healing the present study was undertaken.

Materials and methods

Collection and authentication

The fern *Pteris vittata* L. (PV) was collected from Chandraprabha Vanrai, Dapoli in the Ratnagiri district of Maharashtra in the end of monsoon and was authenticated by Dr. P. G Diwakar from Botanical Survey of

India, Pune under the number BSI/WC/Tech./2011/306. A voucher specimen of the herbarium was submitted in APT Research Foundation, Pune.

Preparation of plant extracts

The fronds were cleaned and shade dried in a dryer and the dried fronds obtained were crushed and every time 100 g of dried fronds were weighed and kept in the thimble of Soxhlet apparatus and extracted with 300 ml of water and ethanol respectively for 24 h each (8 h/day for 3 days) for obtaining aqueous and ethanolic extract. The aqueous as well as ethanolic extracts obtained were concentrated in rotary evaporator under vacuum and analyzed for extractive yield and ash values.

Chemicals and reagents

A 5% povidone iodine (PI) solution under the brand name "Cipladine" was obtained from Cipla Pvt. Ltd. Streptozotocin (STZ) was procured from Sisco Research Laboratories Pvt. Ltd. and TRI reagent was from Sigma Aldrich, St. Louis, USA. Ketamine and xylazine were procured from Aquafine injecta Pvt. Ltd. and Indian Immunologicals Ltd. respectively All other chemicals used for the study were of analytical grade.

Isolation and characterization of bioactives

In our previous study both aqueous and ethanolic extracts of PV showed adequate potential in producing hypoglycaemic conditions in vivo and *invitro.* In the ABTS (2, 2′ - azino-bis (3-ethylbenzo thiazoline – 6-sulfonic acid) antioxidant assay, the 500 μg of aqueous extract displayed 82% free radical scavenging activity to that of ethanolic extract which was 84%. HPTLC profile of both the extracts of PV was also similar with slightly more bands in the ethanolic extract. Quantification of polyphenols spectrophotometrically revealed that flavonoids present was 6% higher in the ethanolic extract as compared to aqueous extract and the phenolic content was 29% higher in the ethanolic extract as compared to aqueous extract [25]. Subsequently, both the extracts was subjected to TLC and ethanolic extract was found to be well separated than the aqueous extract, therefore, for ease of isolation and characterization ethanolic extract was further subjected to isolation of actives by column purification using Hexane: Chloroform: Methanol at a ratio of 30:60:1. Followed by this, HPLC was performed on a Shimadzu LC-20 which was equipped with a model UV-Vis detector SPD-10. The Fractions obtained after column chromatography were tested for efficacy and the fraction that showed good glucose uptake in *invitro* pancreatic β cell assay was subjected to FTIR analysis in Shimadzu Model 8400S with DRS attachment using dried Potassium Bromide (KBr) salt disc. A 50 mg of column purified samples were dissolved in deuterated chloroform ($CdCl_3$) and placed in the Varian NMR 300MHZ for H^1 and C^{13} Spectrum. Later, the spectrums were detected by VNMRJ software and the chemical shifts were noted as ppm (parts per million). Lastly, for Mass spectroscopy, 20 mg of the column purified plant fraction was dissolved in chloroform and was analyzed through Shimadzu GCMS-QP 2010 Plus.

Animals

Male Wistar rats (180-200 g) were procured from National Toxicology Centre, Pune. In the present study the animals were distributed into following four groups of 6 rats each – Group I-diabetic control, Group II-PI treated, Group-III-ethanolic extract of PV treated and Group IV-aqueous extract of PV treated rats. Animals were housed under standard environmental conditions of temperature (23 °C) and 12 h light and dark cycle. All the animals were provided with food and water ad libitum. The study protocol was approved by Institutional Animal Ethics Committee of National Toxicology Centre, Pune through Research Protocol no.160 and executed by following the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines.

Induction of hyperglycemia

Experimental hyperglycemia was induced by injecting STZ (40 mg/kg b.w) in ice cold citrate buffer intraperitoneally (i.p) to the overnight fasted rats [26]. On the 5th day of induction, the animals were bled through the retro orbital plexus and blood was collected in heparinised tube. The plasma glucose was measured in a biochemical analyzer by Glucose oxidase-peroxidase (GOD/POD) method. The animals that developed more than 300 mg/dl of plasma glucose on the 5th day and those rats who maintained blood glucose level > 250 mg/dL for 2 weeks after hyperglycemia induction were deemed diabetic and used for the experiment [27].

Diabetic excision wound model

The animals were anesthetized by injecting intramuscularly ketamine hydrochloride and xylazine in 1:1 concentration. The dorsal fur of the animals was shaved with electric clipper. Using toothed forceps and pointed scissors circular excision wound of 300 to 400 mm^2 were created to full thickness [28]. Wound areas were measured by tracing the wound on transparent sheet with permanent marker by using millimeter based graph paper on days 0, 3rd, 6th, 9th, 12th and 15th for all groups [29]. A 2% aqueous and ethanolic extracts were made by dissolving 200 mg of extracts in 10 ml of water and from that 100 μl of the solution was applied topically to each rat for 15 days once daily on the wounded site. The control group of animals were not treated with

any drug and wounds were kept open [30]. Whereas the PI treated group of animals were applied with 5% solution of PI [31]. Percentage of the reduction in wounded area was calculated from wound contraction. Tissue biochemical parameters, gene expression and histopathological examination were carried out by using tissue specimen isolated from the healed skin of each groups of rat [32].

Biochemical parameters

Circular wound area was excised and evaluated for various biochemical parameters for evaluating the healing properties of PV.

Estimation of hydroxyproline

Healed tissue was excised and dried in glass vials in a 110 °C oven for 48 h. A 5 mg of lyophilized sample was hydrolyzed with 5 ml of 6 N HCl at 110 °C for 18 to 20 h in a sealed tube for estimation of hydroxyproline [33].

Estimation of collagen

The hydroxyproline content may be converted to its equivalent collagen through multiplication by the factor 7.46 [34].

Estimation of hexosamine

It was carried out as per the protocol given briefly, 5 mg of lyophilized tissue sample was hydrolyzed with 5 ml of 2 N HCl at 110 °C for 6 to 7 h., evaporated to dryness and the residue was dissolved in known amount of water. The solution was treated with 1 ml of freshly prepared 2% acetyl acetone in 0.5 M sodium carbonate and boiled for 15 min. After cooling 5 ml of 95% ethanol, 1 ml of Ehrlich's reagent was added and mixed thoroughly. The purple red color developed was read after 30 min at 530 nm spectro photometrically [35].

Antioxidant parameters

Catalase

Estimation of catalase was carried out by taking 10% tissue homogenate in pH 7, a detergent e.g. (1% Triton X-100) was added and it was further diluted with phosphate buffer pH -7 (1:100) and reaction with H_2O_2 was monitored spectrophotometrically [36, 37].

Reduced glutathione (GSH) assay

A tissue homogenate was prepared with 0.5 g of the skin tissue with 2.5 ml of 5% TCA. The precipitated protein was centrifuged at 1000 rpm for 10 min. The supernatant (0.1 ml) was used for the estimation of GSH [38].

Lipid peroxidation (LPO) assay

The assay was done by adding 1 ml of tissue homogenate to 2 ml of TCA: TBA: HCl reagent (15 g TCA, 0.375 g TBA dissolved in 100 ml of 0.25 N HCl.), all tubes were vortexed for few seconds in boiling water bath, cooled to room temperature and centrifuged for 15 min. Supernatants were pipetted out in cuvette and OD was measured at 535 nm against blank [39].

RNA isolation and real time PCR for gene expression

The total RNA was isolated from the tissue samples by using TRI reagent. Precipitation of RNA was done by mixing with isopropyl alcohol (0.5 ml).RNA samples were centrifuged and the pellets were washed with 75% ethanol and later air dried to remove the trace of ethanol. The RNA pellets were dissolved in DEPC-treated water and checked on Spectrophotometer for its qualitative and quantitative analysis.

cDNA preparation

Total RNA (2 μg) was reverse transcribed into first-strand cDNA (ABI kit) following the manufacturers procedure.

Gene expression in real time PCR

The synthesized cDNA (50 ng/μl) was used as a template for polymerase chain reaction (PCR) amplification of five genes namely TNF- α, IL-6, MMP-9, VEGF, and Collagenase, GAPDH was used as internal control. The primers for GAPDH, TNF- α, IL-6, MMP-9, VEGF, and Collagenase are as follows:

GAPDH- F-5'TGAACGGGAAGCTCACTGG3'
GAPDH- R-5'TCCACCACCCTGTTGCAGTA3'
TNF-α- F-5'AGTTGGCTCTGCCAAGTCTCAGAT3'
TNF-α- R-5'TGGCACTCATCAGGATGACACCAT3'
Interleukin-6 F-5'AACTCCATCTGCCCTTCAGGAA CA3'
Interleukin-6 R-5'AAGGCAGTGGCTGTCAACAACA TC3'
MMP-9- F-5'GTGTGGAGTTATGATGATGC3';
MMP-9- R-5'CAATGCGATTACTCCAGAT3';
*VEGF*1- F-5'GACCCTGGTGGACATCTTCCAGGA3';
*VEGF*1- R-5'GGTGAGAGGTCTAGTTCCCGA3';
Collagenase-2 F-5'GTCGATTCACCTACAGCACG3';
Collagenase-2 R-5'GGAGTTTACACGAAGCAGGC3'

Real time PCR was performed using step one Real time PCR system (ABI). PCR were carried out for 45 cycles using the following conditions: denaturation at 95 °C for 45 s, annealing at 62.7 °C for 30 s, and elongation at 72 °C for 15 s. The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene GAPDH and the samples were analyzed in triplicates. Melting curve analysis was performed to assess the specificity of the amplified PCR products. A

dissociation curve analysis of all primers showed a single peak.

Histopathology

Tissue fixed in 10% formalin was used and was embedded in paraffin wax. Serial sections of paraffin embedded tissues were made. Staining was done by using Haematoxylin and Eosin, which were examined by light microscope.

Qualitative and quantitative analysis of cellular details of skin tissue consisting epidermis, dermis and subcutaneous tissue were observed microscopically at an average of 5 sites by a veterinarian histopathologist. Congestion, edema, vascularization, necrosis and epithelialization were assessed. Presence of cell types like polymorpho nuclear leukocytes, mononuclear cells, fibroblasts were also quantified in the skin tissues. Overall grade score used were as follows- NAD = No Abnormality Detected, Minimal changes (+ 1), Mild changes (+ 2), Moderate changes (+ 3), Severe changes (+ 4).

Statistical analysis

Results obtained from the wound healing models have been expressed as mean ± SD and were compared with the corresponding control group by one way ANOVA test using Graphpad Prism software version 5 for assessing statistical significance. P values less than 0.05 were considered as statistically significant.

Results

Physico-chemical parameters

The dried fronds of PV were analyzed for some physicochemical parameters which are summarized as follows: The loss on drying was 55% of the wet weight and the extractive values were 21% for aqueous extract and 18% for ethanolic extract. The total ash was 17%, of which acid insoluble ash was 3% and water soluble ash was 14%.

Excision wound model

In the excision wound model, there were non-significant changes in the wound diameter of all groups on day 0 as compared to diabetic control rats. The treatment started from the day of wound creation to respective groups for 15 days while wound of diabetic control rats were kept untreated. The 15th day wound area reduction was found to be statistically significant in PI treated rats and both the extract of PV treated rats as compared to diabetic control rats with $P < 0.0001$ when analyzed by one way ANOVA using Bonferroni's multiple comparison test.

Similarly, there was statistically significant decrease in the wound area in both the extracts of PV treated rats as compared to PI treated rats on the last day of treatment ($P < 0.0001$). However, there was no significant changes observed between PV aqueous treated wound area versus PV ethanolic extract treated wound area on day 15. Animals treated with aqueous extract of PV showed 87% reduction in the wound area while the ethanolic extract treated group showed 85% reduction in the wound area when compared with 0 day data. The percentage of wound reduction was found to be 79% in PI treated rats which were only 65% in untreated diabetic rats when compared with 0 day data. The wound reduction percentage when compared to diabetic control was 22% in the PI treated group whereas the wound reduction percentage in ethanolic and aqueous extract treated rats were 30% and 33% respectively as compared to diabetic control rats (Fig. 1).

Hydroxyproline, collagen and hexosamine assay in wound tissue

A significant increase of 52% in the hydroxyproline content was observed in the PI treated group as compared to diabetic rats. Similarly 69% and 71% increase in hydroxyproline content was observed in ethanolic and aqueous extract treated rats respectively.

Generally an increase in hydroxyproline content is ultimately responsible for increase in collagen levels. In the present study PV extracts and PI treated rats showed 30–40% increase in collagen content as compared to diabetic rats. The two test extracts treatment to diabetic rats revealed a significant increase of 67% and 74% in hexosamine and the PI treated rats showed increase in the hexosamine content by 57% as compared to diabetic rats. The values were statistically significant at $P < 0.001$ when compared to untreated diabetic control group (Table 1).

Tissue antioxidant parameters

There was a significant increase of 50–60% in the catalase content in the PI treated, aqueous extract and ethanolic extract treated groups which were much higher than disease control group. On the other hand the GSH content has increased by almost 40% in the treated rats as compared to the disease control in the present study, the LPO content was much higher in diabetic control group which was 251.70 ± 19.31 U/gm of tissue. But in the PI treated group as well as both the test extract treated groups, the LPO content was found to be reduced by 53–63% as compared with the diabetic control group (Fig. 2).

Gene expression in wound tissue

The MMP-9, Collagenase-2 and VEGF-1 genes were down-regulated in disease control rats compared to normal. The PI treated group showed 3.2 fold increase in the MMP-9 expression whereas the PV ethanolic and

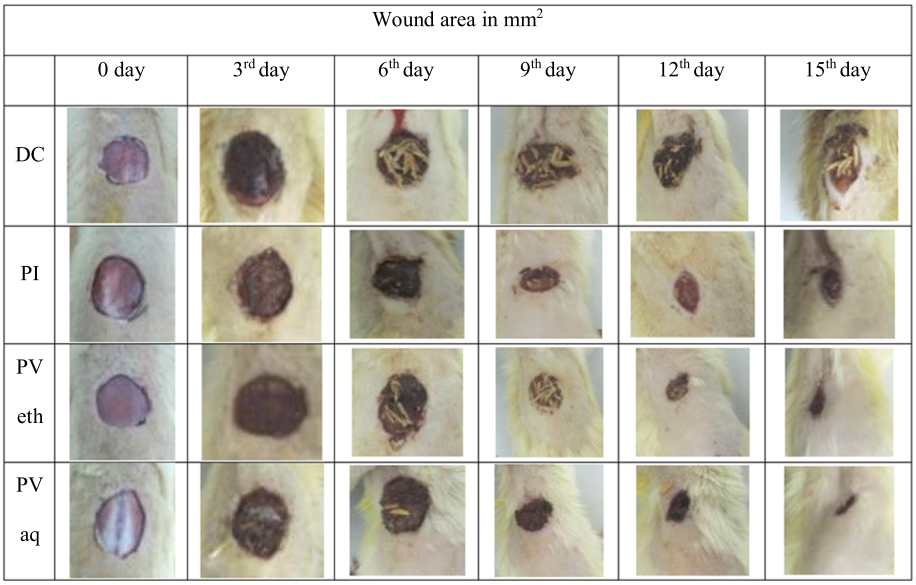

Fig. 1 Representative images of wound contraction in diabetic rats. Representative images showing the wound contraction pattern at 3 days interval measured in mm^2 and photographed from a fixed distance tripod stand

PV aqueous a group showed 2.8 and 2.4 fold increase in the mRNA expression of MMP-9 respectively.

In the Collagenase-2 gene expression there were 4.7, 4.4 and 4.0 fold increase observed in PI treated rats, PV ethanolic and PV aqueous extract treated rats respectively. Similarly, statistical significant increase of 7.5, 5.5 and 5.8 fold increase in the expression of VEGF-1 gene was evident in the PI treated rats and both the extract treated rats respectively. The genes associated with inflammation like TNF-α and IL-6 were up-regulated in disease control rats compared to normal rats, indicating increase in cytokine secretion which results into inflammation.

Conversely in the PI treated rats and PV ethanolic and aqueous extract treated rats the expression of genes associated with inflammation were notably down regulated. The down regulation of TNF-α was 5.0, 4.8 and 5.1 fold in the treated rats. Likewise, PI treated rats and PV ethanolic and aqueous extract treated rats significantly suppressed the mRNA expression of IL-6 by 5.6, 4.7 and 7.0 fold respectively (Fig. 3).

Histopathological observations

The histopathology of the wound area in diabetic rats showed incomplete scab formation with hemorrhagic changes in the epidermal layer. Mild inflammatory reaction in both epidermal and dermal layer was also evident with infiltration of neutrophils. Poor proliferation of fibroblast with formation of granulation tissue was also seen indicating delay in healing process.

Treatment of rat wounds with plant extract of PV led to reduced inflammatory changes and absence of hemorrhages in the epidermal layer. Re-epithelialization of skin was observed as an indicator of complete healing and scab formation was also almost complete. Dermis layer and subcutaneous tissue showed prominent collagen formation with proliferation of fibroblast tissue and formation of new blood vessels in the healing area (Fig. 4). In the PI treated animals scab formation and healing was not fully achieved and hence formation of new epidermal skin epithelial tissue and complete covering of the wound area was somewhat lacking.

Table 1 Wound tissue biochemistry data in diabetic rats

Treatment	Collagen (μg/gm)	Hydroxyproline (μg/gm)	Hexosamine (mg/gm)
DC	240.21 ± 17.64	32.20 ± 2.36	6.38 ± 0.74
PI	365.04 ± 38.81***	48.93 ± 5.20***	14.95 ± 3.23***
PV Eth	405.32 ± 30.09***	54.33 ± 4.03***	19.23 ± 1.69***
PV Aq	411.54 ± 31.09***	55.16 ± 4.16***	24.35 ± 1.75***

Values are expressed as Mean ± SD ($N = 6$) *** $P < 0.001$ when compared with disease control group by one way ANOVA by Dunnett's Test

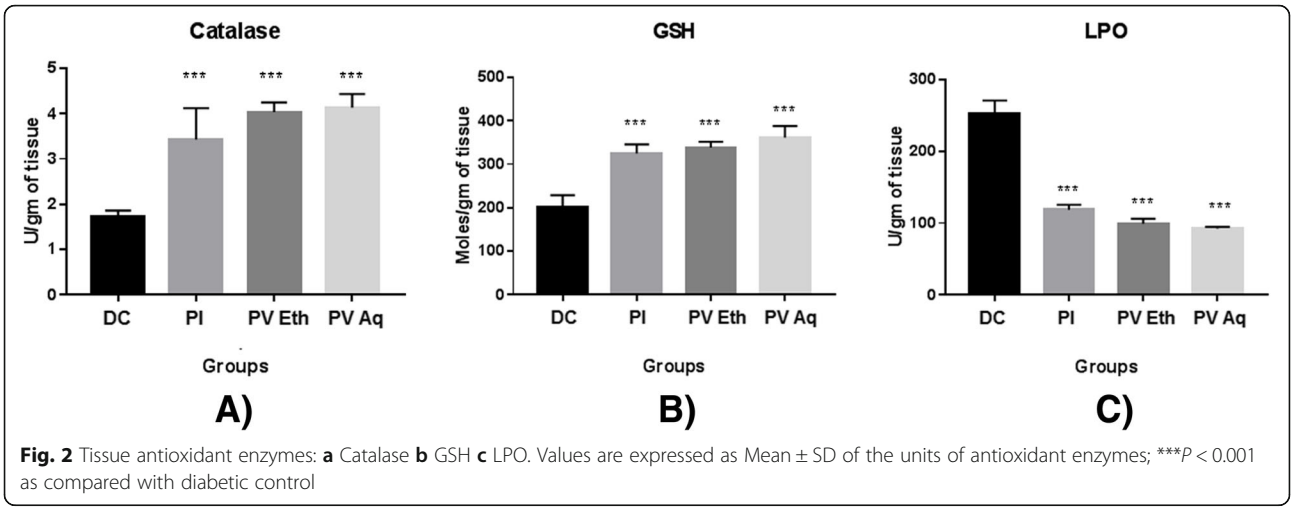

Fig. 2 Tissue antioxidant enzymes: **a** Catalase **b** GSH **c** LPO. Values are expressed as Mean ± SD of the units of antioxidant enzymes; ***$P < 0.001$ as compared with diabetic control

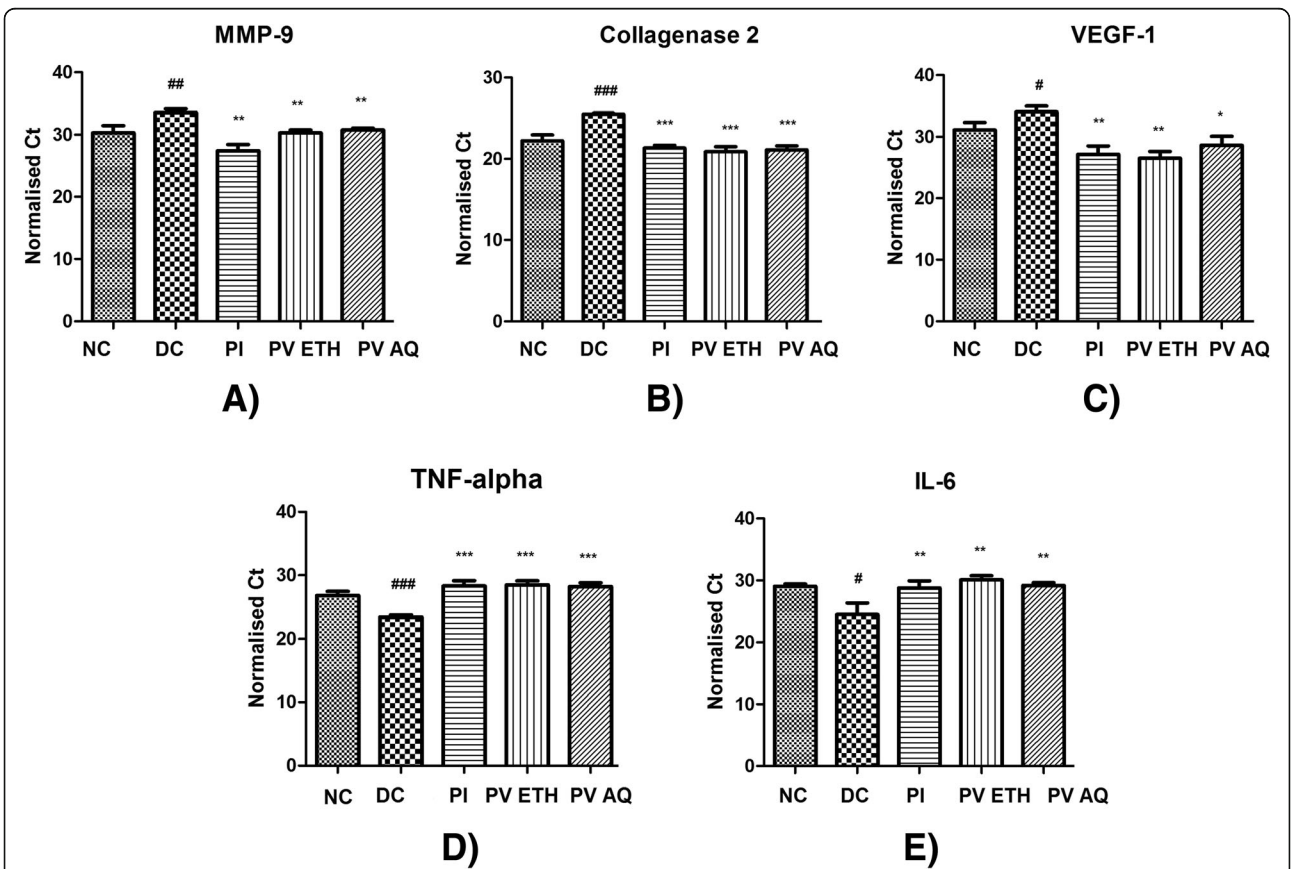

Fig. 3 Tissue Gene Expression data: **a** MMP-9, **b** Collagenase 2 **c** VEGF-1, **d** TNF-α and **e** IL-6. Values are expressed as Mean ± SD of the normalised Ct values; ###$P < 0.001$(compared with normal control), *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ (compared with diabetic control). NC: Normal control (non wounded tissue); DC: Diabetic control; PI: povidone iodine; PV ETH: Ethanolic extract of PV; PV AQ: Aqueous extract of PV

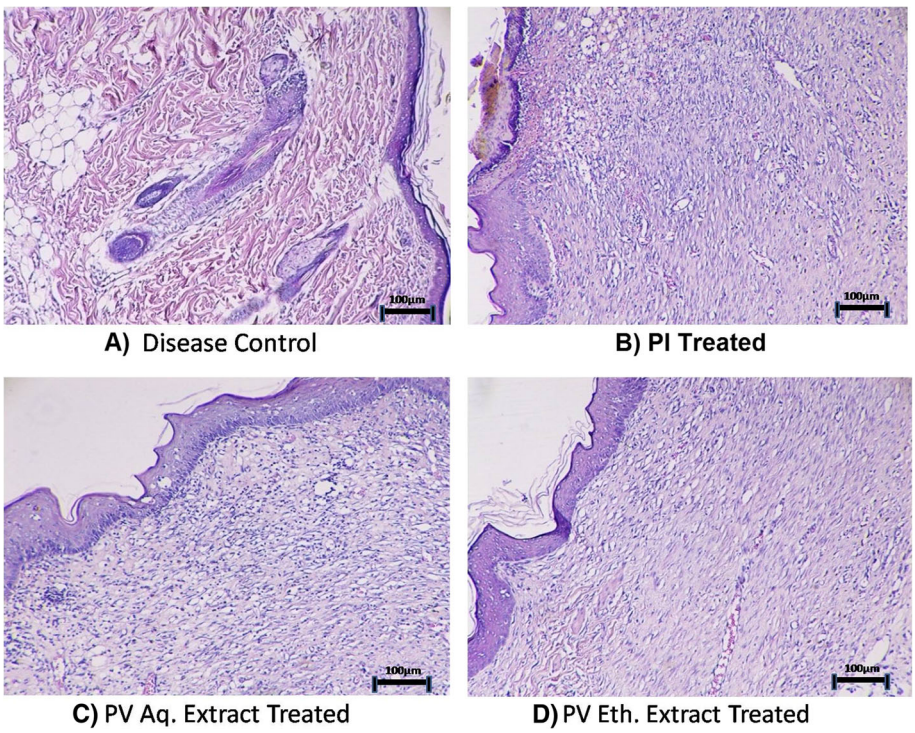

Fig. 4 Histopathological observation of the skin patch of the diabetic rats. Representative microphotographs of skin histopathology at 100X magnification showing healed outer contour of skin tissue in (**b**, **c** and **d**) as compared to incomplete healing of (**a**)

Isolation and characterization of bioactives

The process involved for isolating bioactives from ethanolic extract of PV involved column purification into 7 bands. The separated bands were tested for bioefficacy and ultimately, Fraction 1 of PV was selected for UV-Visible fingerprinting, HPLC, FTIR, C^{13} and H^{1}-NMR and ultimately mass spectroscopy for obtaining bioactives (Fig. 5).

Discussion

In the present investigation, efforts have been made to focus on wound healing property of ethanolic and aqueous extract of PV in hyperglycemic conditions. Known worldwide for its arsenic hyperaccumulation, this Chinese brake fern has many therapeutic effects. Most importantly in our previous study [24], it was seen that despite its inherent property of accumulating harmful toxic metals like arsenic, chromium, and cadmium through its roots, the aqueous extract of its fronds were safe at 2000 mg/kg when administered orally to mice and rats. Repeated administration of its aqueous extract for 28 days orally also did not produce any adverse toxic symptoms. Hyperglycemia induced by STZ was maintained during the present study as evident from the blood glucose tests carried out before and after study completion. Apparently, investigating the wound size of the rats it is clear that epithelialization of the wound area was lesser in diabetic rats but substantial hastening of epithelialization was seen in both the extracts of PV treated rats which was as good as PI treated rats. Another aspect of faster wound healing by PV can be increase in collagen turnover as it is a major protein of the extracellular matrix which ultimately contributes to wound strength. Hydroxyproline and hexosamine being the index of collagen turnover was found to be increased by PV extracts leading to rapid wound healing and the results obtained were comparable to that shown by standard marketed drug-PI. It indicated that PV help in crosslinking glycosaminoglycan chains from repeating hexosamine units which in turn support collagen mesh for wound healing. It is well known that delayed wound healing is associated with ROS therefore, removal of free radicals by antioxidant enzymes play key role in wound bed. The PI treated group showed sufficient antioxidant activity in the wound tissue by scavenging of free radicals and minimizing the oxidative stress. Similarly, both the extracts of PV were competent in maintaining the redox balance by augmenting the anti-oxidant enzymes like catalase and GSH and minimizing the LPO in wound site.

From the above findings, it is evident that throughout the study, may it be wound reduction percentage, tissue biochemistry, antioxidant assays, histopathology or gene

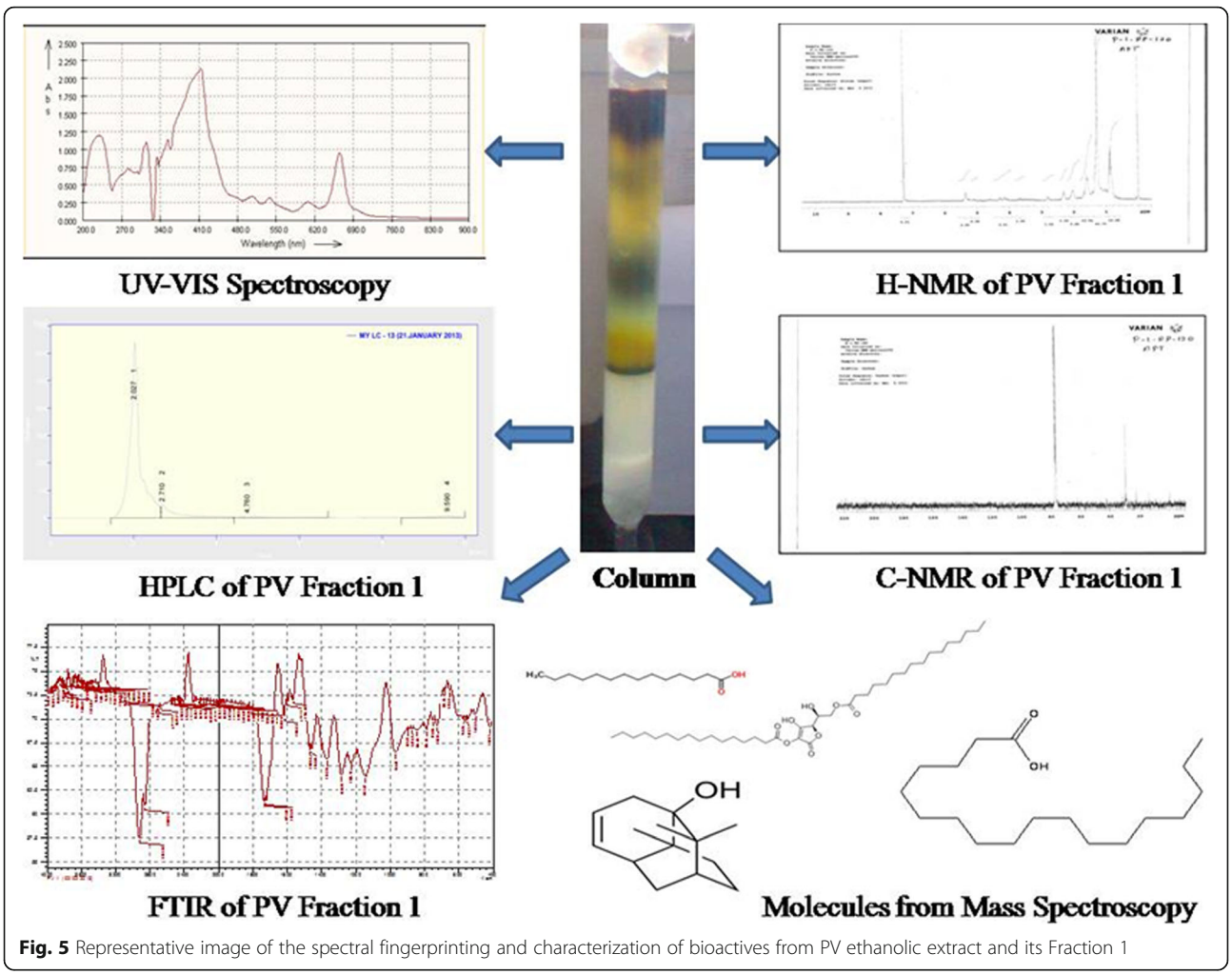

Fig. 5 Representative image of the spectral fingerprinting and characterization of bioactives from PV ethanolic extract and its Fraction 1

expression, the highest efficacy was showed by rats treated with aqueous extract followed by ethanolic extract. Both the extracts showed promising wound healing efficacy in hyperglycemic condition which was considerably better than the PI treated rats. The PI treated rats showed wound healing efficacy which was better than untreated diabetic rats but failed to surpass the results of the PV extracts treated rats in all the parameters evaluated.

During the literature search on PV it was found that using RNAi-based gene silencing, other authors demonstrated critical roles for three genes in establishing arsenate tolerance in PV which were PV GAPC1 (Glyceraldehyde 3-Phosphate Dehydrogenase), PV OCT 4 (Organic Cation Transporter 4) and PvGSTF1 Glutathione S- Transferase) [40].

Therefore, it can be hypothetized that GAPDH in PV might help reduce glucose in the diabetic conditions by catalyzing the sixth step of glycolysis pathway. The OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3), are located in liver, kidney and intestine in humans and are responsible for absorption, distribution and elimination of xenobiotics [41] but PV has an OCT 4 gene which help to convert arsenate to form arsenite that is ultimately transported into the vacuole probably making it safe for consumption. Lastly the additional presence of PV-GSTF1 (Glutathione S- Transferase) gene in PV might contribute to fasten the process of wound healing by destroying the free radicals in treated rats which was somewhat impaired in diabetic rats. Glutathione S- Transferase is for its major defense processes which combat the deleterious oxidation effects resulting from ROS [42].

It is already reported that in diabetes delay in influx of inflammatory cells into a wound site initially and later prevention of deposition of matrix components leads to interaction of advanced glycation endproducts (AGE) with its receptors and triggers release of pro-inflammatory molecules such as TNF-a and MMPs which limit wound healing [43].. In the present study, the extracts of PV upregulated MMP-9, Collagenase-2 and VEGF-1 whereas downregulated TNF- α and IL-6 and thereby reduced inflammation. Similar trend in

modulation of the above genes were also observed in PI treated rats which is evident from the significant increase in mRNA expression of MMP-9, Collagenase-2 and VEGF-1 and suppression of the mRNA expression of TNF- α and IL-6 as compared to diabetic group. Although controversial role of MMP-9 persist, increase in MMP-9 gene expression in this case can be attributed to keratinocytes which at the leading edge of the wound promotes cell migration and re-epithelialization and our findings tally with the previously published reports [44–46]. It is also believed that at the later stage of post wounding, a tissue remodeling phase begins; where a state of equilibrium is reached between collagen synthesis and destruction [47]. With the increase in VEGF expression, it is assumed that PI and both the extracts of PV promote angiogenesis by stimulating VEGF in skin wounds in diabetic rats.

In our previous study, quantification of flavonoids, phenols and the HPTLC profile of PV were reported along with antioxidant assays [25]. From the comparative analysis of all the results obtained from in vivo and in vitro assays it was evident that both aqueous and ethanolic extracts have concurrent efficacy in maintaining the glucose homeostasis and also wound healing capacity. However, only ethanolic extract was taken forward for isolation of actives due to its slightly better antioxidant capacity and presence of more flavonoids and phenol content in it.

In the column chromatography, the ethanolic extract of PV separated into seven fractions. The first fraction was deep green in colour followed by a yellow band, green band, light yellow band, light green band, light yellow band and lastly a dark brown band. The UV- visible spectroscopy deals with electronic transition and gives information mainly about multiple bonds and conjugation. The PV ethanolic and aqueous extracts have shown lambda max at 323 and 327 nm respectively. The UV spectrum of Quercitin has shown a maximum peak at 325 nm and Gallic acid at 305 nm.

FTIR spectroscopy provides the vibration of bonds and gives information about the functional groups present and how atoms are bonded together [48]. The column purified fraction which showed best in vitro glucose uptake efficacy in the pancreatic cells were subjected to FTIR analysis. In the FTIR spectrum, the PV fraction have showed peak at the 684 cm^{-1} which indicated presence of C-H. Another peak at 1367 cm^{-1} could be due to presence of aromatic rings and a peak at 1616 cm^{-1} may correspond to phenyl group. Peaks at 1733 cm^{-1} could be due to a C= O stretch group and the presence of OH groups were evident from peaks at 3390-3514 cm^{-1}.

Through the study of NMR spectroscopy the number and environments of all the hydrogen and carbons in a molecule can be assessed. In the present study, both H-NMR and C NMR were done which have shown the characteristic chemical shifts and their corresponding ppm. Reviewing of the available references for chemical shifts it was found that 4.1–4.3 ppm corresponds to the presence of phenolic group. Moreover, ppm at 1.300–1.500 corresponds to the presence of aliphatic compounds. Acidic group was assumed to be present owing to the presence of peak at 2.300–2.400 in the PV fractions. Peaks at the range 2.200 indicated the presence of esters in the fractions. C_{13} NMR data is used to complement H_1 NMR data, and is particularly useful at establishing the type of groups present in the samples molecules by comparison with compiled data. From the C-NMR studies it was found the CH3 occur at lower ppm, CH2 occur at mid ppm and CH occur at higher ppm.

MS has proved to be one of the most effective techniques in biomedical research, in special when complex matrixes of biological samples must be analyzed. It plots relative abundance versus mass /charge ratio. Each line upon the mass spectrum indicates the presence of particular atoms or molecules of a particular mass. The most intense peak in the spectrum is taken as base peak. Its intensity is taken as 100 and other peaks are compared with it.

GC-MS analysis of fraction 1 of PV led to the elucidation of eight to ten compounds, which were both aliphatic and aromatic in nature. The molecular formula and weights of the compounds isolated from PV were mainly 3-Hexadecene ($C_{16}H_{32}$; MW: 224), Norpatchoulenol ($C_{14}H_{22}0$; MW: 206), Myristic acid ($C_{14}H_{28}O_2$; MW:228.37), Labdane $C_{20}H_{38}$; MW: 278.51), Octadecanoic acid/ stearic acid ($C_{18}H_{36}O_2$; MW: 284.48), Oleic acid $C_{18}H_{34}O_2$;MW: 282.46, L-Ascorbyl 2,6-Dipalmitate $C_{38}H_{68}O_8$; MW: 652). Among these, Myristic acid [49], Stearic and Oleic acids are known to be associated with modulation of immune response in wound healing [50] and these fatty acids accelerate the inflammatory phase of wound healing [51]. L-Ascorbyl 2, 6-Dipalmitate (Ascorbic acid) one of the active compounds present is reported to be responsible for the antioxidant activity. Moreover, the GC-MS analysis confirmed the presence of polyphenols, which might be responsible for its anti hyperglycaemic condition in vivo and in vitro.

Conclusion

Thus, it can be concluded that the lithophytic fern, PV not only eliminates arsenic from the contaminated environment but it can also modulate the microenvironment inside endothelial and epithelial cells. Firstly, both the extracts contribute to wound contraction by augmenting collagen turnover. Secondly, it modulate the antioxidant molecules which clears the wound site from free radical oxidative damage. Thirdly, upregulates

MMP-9 Collagenase-2/MMP-8 and VEGF-1 and downregulates TNF- α and IL-6 leading to reduction in inflammation and helps in generating new blood vessels.

Summarizing all these factors, both aqueous and ethanolic extract of PV proves its candidature for being an alternative approach in wound healing regime by proper isolation of its active components. Our previous study on PV revealed the presence of flavonoid and phenolic compounds [25] and in the present study the FTIR, NMR and GC-MS of the probable fraction showed presence of polyphenolic compounds, Myristic acid, Oleic acid, Stearic Acid, Calcipotriol, Norpatchoulenol, L-Ascorbyl 2, 6-Dipalmitate predominantly. These polyphenolic compounds might be responsible for the faster wound healing efficacy in the hyperglycemic conditions; however, extensive studies are warranted for isolation and characterization of bio-active components responsible for its astringent, anti-microbial and healing property in chronic diabetic wounds.

Abbreviations

ANOVA: Analysis of Variance; SD: Standard Deviation; PV: *Pteris vittata. L*; CPCSEA: Committee for the Purpose of Control and Supervison of Experiments on Animals; STZ: Streptozotocin; DC: Disease Control; PI: Povidine Iodine; Aq: Aqueous Extract; Eth: Ethanolic Extract; CAT: Catalase; GSH: Reduced Glutathione; LPO: Lipid Peroxidation; MMP: Matrix Metaloproteins; VEGF: Vascular endothelial Growth Factor; TNF- α: Tumor Necrosis Factor-alpha; IL-6: Interleukin 6; TCA-TBA-HCl: Trichloro Acetic acid: Thiobarbituric acid: Hydrochloric acid

Acknowledgements

The authors are thankful to Dr. K.G. Apte of National Toxicology Centre for providing the in-house Animal facility for conducting the research. We are also thankful to Dr. Avinash M. Pradhan of KEM Hospital Research Centre for conducting the histopathology and analyzing the findings. We are extensively thankful to Mr. Sushant Shengule, for gene expression, Dr. Asha Thomas, DY Patil College, Pune, for proving facility for FTIR analysis. Dr. Sheetal Chaudhari, Sophisticated Analytical Instrumentation Facility, University of Pune, for H1 and C13 NMR and Dr. M.P. Sathinarayanan, Bombay Textile Research Association, for GC-MS analysis.

Authors' contributions

TSP conducted the experiment, analyzed the data and drafted the manuscript. BBD performed the procurement of plant material and conducted the biochemical gene expression and antioxidant parameters and was a major contributor in writing the manuscript. YPT interpreted the results obtained and co- conducted the experiments. SB approved the study design and helped in the isolation and characterization of bioactives associated with this study. All authors read and approved the final manuscript.

Funding

The study was self funded for design, collection, analysis, interpretation of data and writing the manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by Institutional Animal Ethics Committee of National Toxicology Centre under the Research Project No: 160 and conducted by following the CPCSEA guidelines.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]APT Research Foundation, DSIR-SIRO Recognized Research Institute, Sinhagad Road, VadgaonKhurd, Pune, Maharashtra 411041, India. [2]APT Testing and Research Pvt. Ltd. (Previously National Toxicology Centre), S. No. 36/1/1, M. N. 199, VadgaonKhurd, Sinhagad Road, Pune 411 041, India. [3]Institute for Excellence in Higher Education, Bhopal, Madhya Pradesh 462016, India.

Received: 16 April 2020 Accepted: 3 November 2020
Published online: 23 November 2020

References

1. Jameeluddin M, Janarthan M, Duraivel S. Evaluation of anti-diabetic and wound healing activities of 90% methanolic extract of *Ficus mollis* bark by using wistar strain rats. Ind J Res Pharm Biotech. 2014;2:988–92.
2. Lodhi S, Singhai AK. Wound healing effect of flavonoid rich fraction and luteolin isolated from *Martynia annua* Linn on streptozotocin induced diabetic rats. Asian Pac J Trop Med. 2013;2013:253–9.
3. Estelle E, Nestoras M. Update on management of diabetic foot ulcers. Ann N Y Acad Sci. 2018;1411:153–65.
4. Jeffcoate WJ, Vileikyte L, Boyko EJ, Armstrong DG, Boulton AJM. Current challenges and opportunities in the prevention and management of diabetic foot ulcers. Diab care. 2018;41(4):645–52.
5. Nayak BS, Sandiford S, Maxwell A. Evaluation of the wound healing activity of ethanolic extract of *Morinda citrifolia* leaf. Evid Based Complement Alternat Med. 2009;6:351–6.
6. Shankaran V, Brooks M, Mostow E. Advanced therapies for chronic wounds: NPWT, engineered skin, growth factors, extracellular matrices. Dermatol Ther. 2013;26:215–21.
7. Kunkemoeller B, Kyriakides TR. Redox signaling in diabetic wound healing regulates extracellular matrix deposition. Antioxid Redox Signal. 2017;27:823–38.
8. Sanchez MC, Lancel S, Boulanger E, Neviere R. Targeting oxidative stress and mitochondrial dysfunction in the treatment of impaired wound healing: a systematic review. Antioxidants. 2018;7:1–14.
9. Sathiyaraj G, Muthukumar T, Ravindran KC. Ethnomedicinal importance of fern and fern allies traditionally used by tribal people of Palani Hills (Kodaikanal), Western Ghats, South India. J Med Herbs Ethnomed. 2015;1:4–9.
10. Bondada B, Tu C, Ma L. Surface structure and anatomical aspects of Chinese brake fern (*Pteris vittata*; Pteridaceae) for Pteris china brake fern. Brittonia. 2006;58:217–28.
11. Hall JB. *Pteris vittata* Linn. A gold mine fern in Ghana. Niger Fd. 1970;35:1–9.
12. Ma LQ, Komart KM, Tu C, Zhang W, Cai Y, Kennelly ED. A fern that hyperaccumulates arsenic. Nature. 2001;409:579.
13. Singh M, Govindarajan R, Rawat AKS, Khare PB. Antimicrobial flavonoids rutin from *Pteris vittata* L. against pathogenic gastrointestinal microflora. American Fern J. 2008;98:98–103.
14. Gong XL, Chen ZH, Liang NC. Advances in study on chemical constituents and pharmacological activities of plants of genus Pteris. Zhongguo zhong yao za zhi. 2007;32:1382–7.
15. Benjamin A, Manickam VS. Medicinal pteridophytes from the Western Ghats. Ind J Trad Know. 2007;6:611–8.
16. Salatino MLF, Prado J. Flavonoid glycosides of Pteridaceae from Brazil. Biochem Syst Ecol. 1998;26:761–9.
17. Baskaran X, Jeyachandran R. Evaluation of antioxidant and phytochemical analysis of *Pteris tripartita* Sw. a critically endangered fern from South India. J Fairy Lake Bot Gard. 2010;9:28–34.
18. Kaur P, Kaur V, Kumar M, Kaur S. Suppression of SOS response in *E. coli* PQ 37, antioxidant potential and antiproliferative action of methanolic extract of *Pteris vittata* L. on human MCF-7 breast cancer cells. Food Chem Toxicol. 2014;74:326–33.
19. Rout SD, Panda T, Mishra N. Ethnomedicinal studies on some pteridophytes of Similipal biosphere reserve, Orissa, India. Int J Med Medic Sci. 2009;1:192–7.

20. Karthik V, Raju K, Ayyanar M, Gowrishankar K, Sekar T. Ethnomedicinal uses of Pteridophytes in Kolli Hills, eastern ghats of Tamil Nadu, India. J Nat Prod Plant Res. 2011;1:50–5.
21. Singh SK, Rajkumar SD. Biodiversity and indigenous use of medicinal ferns in Chandraprabha wildlife sanctuary, Chandauli, Uttar Pradesh. Int J Res Stud Biosci. 2017;5:19–25.
22. Fazli W, Khan T, Shehzad O, Shehzad A, Kim YY. Phytochemical analysis and effects of *Pteris vittata* extract on visual processes. J Nat Med. 2016;70:8–17.
23. Singh M, Malhotra K, Singh C. Phytochemical evaluation and antioxidant activity of different samples of *Pteris vittata* in Doon Valley, Uttarakhand region. Int J Pure App Biosci. 2015;3:296–304.
24. Paul T, Das B, Apte KG, Banerjee S, Saxena RC. Hypoglycemic activity of *Pteris vittata* L. a fern on alloxan induced diabetic rats. Inv Imp Planta activa. 2012;2:88–91.
25. Paul T, Banerjee S. *Invitro* evaluation of α-amylase inhibitory activity & antioxidant potential of *Pteris vittata* L with special reference to its HPTLC profile. Int J Pharm Bio Sci. 2013;4:494–503.
26. Mostafavinia A, Amini A, Ghorishi SK, Pouriran R, Bayat M. The effects of dosage and the routes of administrations of streptozotocin and alloxan on induction rate of type I diabetes mellitus and mortality rate in rats. Lab Anim Res. 2016;32:160–5.
27. Ponrasu T, Suguna L. Efficacy of *Annona squamosa* L in the synthesis of glycosaminoglycans and collagen during wound repair in streptozotocin induced diabetic rats. Bio Med Res Int. 2014;2014:1–10.
28. Patil PA, Kulkarni DR. Antiproliferative agents on healing of dead space wounds in rats. Ind J Med Res. 1984;79:445–7.
29. Diwan PV, Tilloo LD, Kulkarni DR. Influence of *Tridax procumbens* on wound healing. Ind J Med Res. 1982;75:460–4.
30. Gupta N, Jain UK. Investigation of wound healing activity of methanolic extract of stem bark of *Mimusops elengi* Linn. Afr J Tradit Complem. 2011;8: 98–103.
31. Tayade PM, Borde SN, Chandrasekar N, Jagtap SA, Joshi AS. Evaluation of wound healing properties of *Psoreliya corolifolia* Linn in diabetic rats. Pharmacol Online. 2011;1:282–8.
32. Oommen ST, Rao M, Raju CVN. Effect of oil of Hydrocarpus on wound healing. Int J Lepr Other Mycobact Dis. 1999;67:154–8.
33. Agarwal PK, Singh A, Gaurav K, Goel S, Khanna HD, Goel RK. Evaluation of wound healing activity of extracts of plantain banana (*Musa sapientum* var. paradisiaca) in rats. Indian J Exp Biol. 2009;47:32–40.
34. Neuman RE, Logan MA. The determination of collagen and elastin in tissues. J Biol Chem. 1950;186:549–56.
35. Talekar YP, Apte KG, Paygude SV, Tondare PR, Parab PB. Studies on wound healing potential of polyherbal formulation using *in vitro* and *in vivo* assays. J Ayurveda Integr Med. 2017;8:73–81.
36. Aebi H. Catalase *in vitro*. Methods Enzymol. 1984;105:121–6.
37. Ramazan M, Kenan G, Senol D, Fatih A. The effect of pre-injury supplementation with selenium or vitamin E on lipid peroxidation and antioxidant enzymes in burn injury. Turk J Med Sci. 2006;36:141–6.
38. Moron MA, Depierre J, Mannervick B. Levels of glutathione, glutathione s-transferase activities in rat liver. Biochim Biophys Acta. 1979;582:67–78.
39. Chandramohan G, Al-Numair KS, Pugalendi KV. Restoration of altered plasma, erythrocyte and liver anti oxidants levels by 3-hydroxymethyl xyliton in streptozotocin-diabetic rats. Int J Integr Biol. 2009;5:176–81.
40. Cai C, Lanman NA, Withers KA, DeLeon AM, Wu Q, Gribsskov M, Salt DE, Banks JA. Three genes define a bacterial-like arsenic tolerance mechanism in the arsenic hyperaccumulating fern *Pteris vittata*. Curr Biol. 2019;29(10): 1625–33.
41. Xiaomin L, Kathleen MG. Transporters involved in metformin pharmacokinetics and treatment response. J Pharm Sci. 2017;106:2245–50.
42. Muguregowda HT, Kumar P, Govindarama PUE. Wound healing potential of intermittent negative pressure under limited access dressing in burn patients: biochemical and histopathological study. World J Plast Surg. 2018; 7:58–66.
43. Ahmed N. Advanced glycation endproducts-role in pathology of diabetic complications. Diabetes Res Clin Pract. 2005;67:3–21.
44. Martins VL, Caley M, O'Toole EA. Matrix metalloproteinases and epidermal wound repair. Cell Tissue Res. 2013;351:255–68.
45. Parks WC. Matrix metalloproteinases in repair. Wound Repair Regen. 1999;7: 423–32.
46. Krishnaswamy VR, Mintz D, Sagi I. Matrix metalloproteinases: the sculptors of chronic cutaneous wounds. Biochimica et Biophysica Acta (BBA) - Mol Cell Res. 2017;1864:2220–7.
47. Park KS, Park DH. The effect of Korean Red Ginseng on full-thickness skin wound healing in rats. J Ginseng Res. 2019;43:226–35.
48. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complem. 2011;8:1–10.
49. Sofrona E, Tziveleka LA, Harizani M, Koroli P, Sfiniadakis I, Roussis V, Rallis M, Ioannou E. *In Vivo* evaluation of the wound healing activity of extracts and bioactive constituents of the marine isopod *Ceratothoa oestroides*. Mar Drugs. 2020;18:1–15.
50. Cardoso CR, Favoreto S Jr, Oliveira LL, Vancim JO, Barban GB, Ferraz DB, Silva JS. Oleic acid modulation of the immune response in wound healing: a new approach for skin repair. Immunobiol. 2011;216:409–15.
51. Pereira LM, Hatanaka E, Martins EF, Oliveira F, Liberti EA, Farsky SH, Curi R, Pithon-Curi TC. Effect of oleic and linoleic acids on the inflammatory phase of wound healing in rats. Cell Biochem Funct. 2008;26:197–204.

Publisher's Note