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Efficacy of Quercetin and Kaempferol from Methanolic Extracts of *Adiantum lunulatum* Burm. F. and *Polystichum acrostichoides* as Antidiabetic Activity

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In present investigation, the aerial parts of two fern species *viz. Polystichum acrostichoides* (PA) and *Adiantum lunulatum* (AL) were used to evaluate the potent antidiabetic activity. Methanol solvent was used for extraction by soxhlation method followed by LC-MS analysis to identify the presence of compounds in both the extracts. Further, isolation of the major compounds was carried out and characterized by IR, mass and NMR techniques. Thereafter, *in vitro* α-amylase inhibitory activity for both the extracts was determined by comparing with acarbose as standard. Thereafter, α-glucosidase inhibition activity followed by glucose uptake was studied using L6 cell line study. Practical yield for both the fern extracts was calculated and observed *A. lunulatum* extract showed more yield (7 g) than *P. acrostichoides* (3 g). Based on the analytical data, isolated compound was identified as quercetin and kaempferol from the *A. lunulatum* and *P. acrostichoides* extracts, respectively. *A. lunulatum* extract even showed better dose dependent α-amylase inhibition and α-glucosidase inhibition activity than *P. acrostichoides* furthermore, L6 cell line study showed increased glucose uptake by *A. lunulatum* extract than *P. acrostichoides* extract when compared with standard insulin. Based on the findings, it appears that the both *A. lunulatum* and *P. acrostichoides* have the potential to be used as antidiabetic drugs due to presence of major isolated flavonoids *i.e.* quercetin and kaempferol as among the bioactive compounds. Antidiabetic efficacy was dosage dependent and *A. lunulatum* extract was more effective in inhibiting glucose uptake than *P. acrostichoides* extract.

Keywords: *Adiantum lunulatum*, *Polystichum acrostichoides*, α-Amylase inhibitor, α-Glucosidase inhibitor, Glucose uptake activity.

INTRODUCTION

The high prevalence of diabetes mellitus is a well-known fact, particularly in Asia. Pancreatic β-cell failure, insulin resistance and postprandial hyperglycemia are the complicated symptoms of type 2 diabetes mellitus, a non-communicable illness [1]. Modern lifestyle and increasing carbohydrate consumption have led to the disease's persistence as a worldwide health concern and economic burden. Delaying the absorption of glucose by inhibiting carbohydrate-hydrolysing enzymes like α-glucosidase and α-amylase in the digestive tract can help maintain stable and lower blood glucose levels. Skeletal muscles account for around 75% of the body's insulin stimulated glucose uptake [2]. There is an excess of sugar in the blood because skeletal muscle tissue is unable to absorb glucose. This is because diabetes develops when either the body's cells do not react appropriately to insulin or insulin production is inadequate. The primary target of insulin-induced glucose uptake stimulation is skeletal muscle [3]. By increasing the number of functional glucose transport molecules in the plasma membrane, insulin enhances glucose absorption in skeletal muscle. Contractile action can also enhance glucose transport in skeletal muscle. When it comes to glucose transport, insulin and contractile activity have additive effects at their maximum [4].

Plenty of medicinal plants are reported as potent antidiabetic activity but literatures on fern species are scanty and even responsible bioactive compounds are also less. Ferns are a prehistoric species of plants that are found all over the world. They are members of the Pteridophyte group and date back millions of years. Ferns are used by humans for a variety of reasons, including industrial and medicinal ones, they are not as well studied as other plant genera. Therefore, it was worthwhile to carry out an antidiabetic activity for fern species. Presently, *Adiantum lunulatum* Burm. F. and *Polystichum acrostichoides* were selected because of their scanty reports as potent antidiabetic agents in scientific platform. Further,

isolation of the bioactive compounds is also essential to establish the therapeutic efficacy by the responsible compound among the various phytochemicals present in the fern extracts.

Of late, *Adiantum lunulatum* is commonly known as Hanspadi or Walking Maiden hair fern belongs to the family Pteridaceae [5]. It is widely distributed in many parts of India especially hilly areas of South India and even in North along the range of the Himalayas with an altitude of 1000-3000 feet [6]. Traditionally, it is used in bleeding diseases, burning sensation, skin infections, erysipelas, epileptic fits, dysentery and elephantiasis [7]. It has many bioactive compounds *viz.* steroids, flavonoids, phenols, terpenoids, tannins alkaloids, *etc.* and due to that, it shows powerful anti-inflammatory, antimicrobial [8], anticancer and antioxidant effect [9] but no report or scanty report on antidiabetic activity for the said fern.

On other hand, *Polystichum acrostichoides* fern was also selected along with the former fern in the present study. The fern is also known as Christmas fern or wintergreen fern belongs to the family Dryopteridaceae. The fern is distributed in Southern hilly region. Traditionally, the fern is used in the treatment of chills, fevers, pneumonia, hoarseness, stomach and rheumatism [10]. Apart from that, the fern is having antimicrobial, anticancer, curing skin eczema [11] but not much information about antidiabetic activity.

EXPERIMENTAL

Plant materials collection: Central Ayurveda Research Institute, Uttarhalli, Bengaluru's, Botanist Dr. V. Rama verified the authenticity of *Adiantum lunulatum* (Voucher no.: RRCBI-4546) aerial parts obtained from Tirupati's Seshachalam hills. and *Polystichum acrostichoides* (Voucher no.: RRCBI- 0751) as verified by Dr. K. Madhava Chetty, a Plant Taxonomist from SV University, Tirupati, India, after its collection from Talakona forest in Andhra Pradesh.

Extraction process: Aerial parts of two ferns were cleaned by running tap water followed by dried in shade for 15 days. Further, the dried materials were separately crushed to powder using a grinder. The powdered material thus obtained was passed through sieve no. 120 to get coarse powder. The powders (500 g) were further extracted using methanol by soxhlet method for 6 h. After extraction, liquid extracts were filtered and filtrates were evaporated using rotary flash evaporator at 40 ºC. The extracts in viscous state were stored in glass bottle (separately labeled) and stored at 4 ºC in refrigeration condition.

Both the extracts were screened through various chemical tests followed by TLC to identify number of compounds and further LC-MS analysis confirmed number of compounds present in both extracts.

Isolation of compounds: Compound was isolated through column chromatography. About 35 g of methanolic extract fraction was loaded in column. Initially, petroleum ether and ethyl acetate in various proportions (100, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90) followed by ethyl acetate and methanol (100, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90) were run. The process continued with pure methanol until colourless. Elution was collected then dried and weighed. In 100% ethyl acetate (fraction no. 24) and 10% methanol in ethyl acetate (fraction no. 25) multiple active bands as observed in TLC. Fraction no. 25 was subjected for further fractionation and isolation of phytocompounds.

Characterization: Isolated compound was characterized by the IR spectra used by Bruker Alpha TKBR and ATR spectrophotometers. Tetramethyl silane (TMS) was used as an internal standard for the ^{1}H and ^{13}C NMR spectra, which were obtained using a Bruker AV NMR equipment with 5 mm ^{1}H & ^{13}C working at 500 MHZ, respectively. Further, molecular mass of the compounds were determined by mass spectrometry with the condition followed as negative ion mode, atomization gas pressure of 40 psi, dry gas velocity of 9.0 l/min, drying temperature of 350 ºC, ionization voltage of 3,000 V, electro spray ionization (ESI), auto MS^{n} for anion detection and finally scanned in the range of 200~800 *m/z*.

In vitro antidiabetic activity

α-Amylase inhibition assay: A mixture of 500 µL of test samples ranging from 50 to 800 µg/mL as well as acarbose at concentrations of 50, 100, 200, 400 and 800 µg/mL were combined with 500 µL of 0.02 M sodium phosphate buffer containing 0.5 mg/mL of α-amylase. The mixture was then incubated at 25 ºC for 10 min. At prescribed intervals following the pre-incubation, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to every tube. The next step was to incubate the combos for 10 min at 25 ºC. Utilizing 1.0 mL of 3,5-dinitrosalicylic acid colour reagent, the process was halted. Once the test tubes had cooled to normal temperature, they were placed in an incubator set over a pot of boiling water for 5 min. Following the addition of 10 mL of distilled water to the reaction mixture, the absorbance was measured at 540 nm to determine its final concentration. This was followed by determining the percentage of inhibition for both extracts [12].

$$\text{Inhibition } (\%) = \frac{A_{\text{Control}} - A_{\text{Treatment}}}{A_{\text{Control}}} \times 100$$

Inhibitory activity of α-glucosidase: In this analysis, 50 µL of test material and standard concentrations, adding 150 µL of *p*-nitrophenyl glucopyranoside (pNPG) and 100 µL of α-glucosidase to the test and control and then measuring the results. The test blank and control blank were replaced with 100 µL of pNPG and 500 µL of phosphate buffer and then incubated for 20 min for each tube at 37 ºC. All the tubes were treated with 500 µL of sodium carbonate after incubation to stop the reaction. Added 100 µL of reaction mixture into the microtitre plate using a pipette. At 405 nm, the absorbance was recorded using an ELISA reader. Simply swapping out the test sample with the standard yielded the same results. The test and control were run three times, whereas the test blank and control blank were run once each [13].

Uptake of glucose in L6 cells: An effect of both fern extracts the rate of glucose uptake was examined using L6 cells (muscle cells of rodents) that use glucose. At first, L-6 cell monolayers were cultured in a growth medium that included 10% fetal bovine serum, 4.5 g/L glucose, 100 IU/mL penicillin and 100 µg/mL streptomycin. The mixture was supplemented with Dulbecco's modified Eagle's medium (DMEM). In a humidified

incubator with ambient oxygen at 37 ºC and 5% CO_2, the cells were maintained. Further, glucose uptake assay was performed by the following process. The cells were grown on six-well plates and kept in a 37 ºC CO_2 incubator for 48 h. After a semi-confluent monolayer had grown, the culture was refreshed in a 37 ºC CO_2 incubator for 18 h using DMEM without serum but with 0.2% bovine serum albumin. The media was thrown away after 18 h and the cells were once again cleaned with Krebs-Ringer bicarbonate buffer. Then, standard insulin, fern extracts and 1 mg of glucose were introduced to the cells and they were then incubated for 30 min. A milliliter of ice-cold buffer was used to wash the cells three times, halting glucose absorption. The supernatant was then collected for glucose analysis. The cells were lysed using three cycles of freezing and thawing. Cell lysate was collected in order to make an estimation of the glucose content. The final step in calculating the quantity of glucose absorbed was to combine 10 µL of sample with 1 mL of reagent and then incubate the mixture at 25 ºC for 25 min. The sample and standard absorbance at 530 nm against the reagent blank within 60 min was calculated using the following relation [14]:

$$\text{Glucose concentration (mmol/L)} = \frac{A_{sample}}{A_{standard}} \times 55.5 \text{ mg/dL}$$

$$= \frac{A_{sample}}{A_{standard}} \times 100$$

Statistical method: GraphPad Prism 5 was used to conduct the statistical analysis. For triple determinations, all data were given as mean ± SEM.

RESULTS AND DISCUSSION

In present study, two known compounds *viz.* quercetin and kaempferol were isolated from *Adiantum lunulatum* and *Polystichum acrostichoides* extract separately due to their reports as antidiabetic activity in different plant sources [15,16] but for the present selected ferns were not having any reports on isolated compounds and their specific activity. Further, both compounds were confirmed with TLC separation followed by characterized by IR, NMR and mass spectra analysis and the result were as per the literature reported earlier [17-19].

The yield (g) for both ferns showed 7 g and 3 g for *Adiantum lunulatum* and *Polystichum acrostichoides*, respectively. Furthermore, extracts were run with TLC for separation of various spots using chloroform, ethyl acetate and acetone (2:1:2) as solvent system.

LC-MS study: The LC-MS analysis (Fig. 1a-b) resulted with the retention time (R_t) and intensity of the compounds and few major bioactive compounds were identified. Rosmarinic acid was eluted at 1.71 min with a mass of 360 *m/z*. Quercetin eluted at 22.15 min (mass of 302.23 *m/z*) and quercetin-3-rhamnoside with mass of 448.38 *m/z* (eluted at 7.23 min) and quercetin-3–0-arabinoside with a mass of 435.35 *m/z* (at 7.95 min). Linolenic acid (280.44 *m/z*) was eluted at 21.03 min. The identified compounds were based on their structure and molecular mass with the degree of similarity and also based on the compound structure reported in the previous reports.

Fig. 1b showed presence of some important bioactive compounds *viz.* eluted first compound was gallic acid with retention time (R_t) of 0.65 (mass of 170.08 *m/z*), followed by epigallocatechin with mass of 305.10 *m/z* at 4.43 min. Kaempferol was eluted at 6.29 with mass of 286.14 *m/z* and derivative of kaempferol *i.e.* kaempferol 3-*o*-(6-malonyl-glucoside) eluted at 10.56 min with mass of 535. 27 *m/z* followed by rutin eluted at 11.06 min with mass of 607.29 *m/z*.

From LC-MS data, further quercetin and kaempferol were isolated from *A. lunulatum* and *P. acrostichoides* plant extracts which are responsible for various therapeutic activity especially antidiabetic activity. Isolated compounds were confirmed with TLC followed by IR, NMR and mass analysis. Toluene and ethyl acetate were used as TLC solvent with the ratio of 7:3.

IR study: Fig. 2a revealed the FTIR spectral analysis of quercetin isolated from *A. lunulatum* extract. It showed 3410.24 cm^{-1} (–OH *str.*) of phenol, 1610.70 cm^{-1} (C-C Ar *str.*), 1265.37 cm^{-1} (C-O *str.*) of aryl ether, 1103.84 cm^{-1} (C-O-C bend.) of ketone, 822.28 cm^{-1} (C-H bend.) of aromatic hydrocarbon. The FTIR spectrum of isolated kaempferol from *P. acrostichoides*

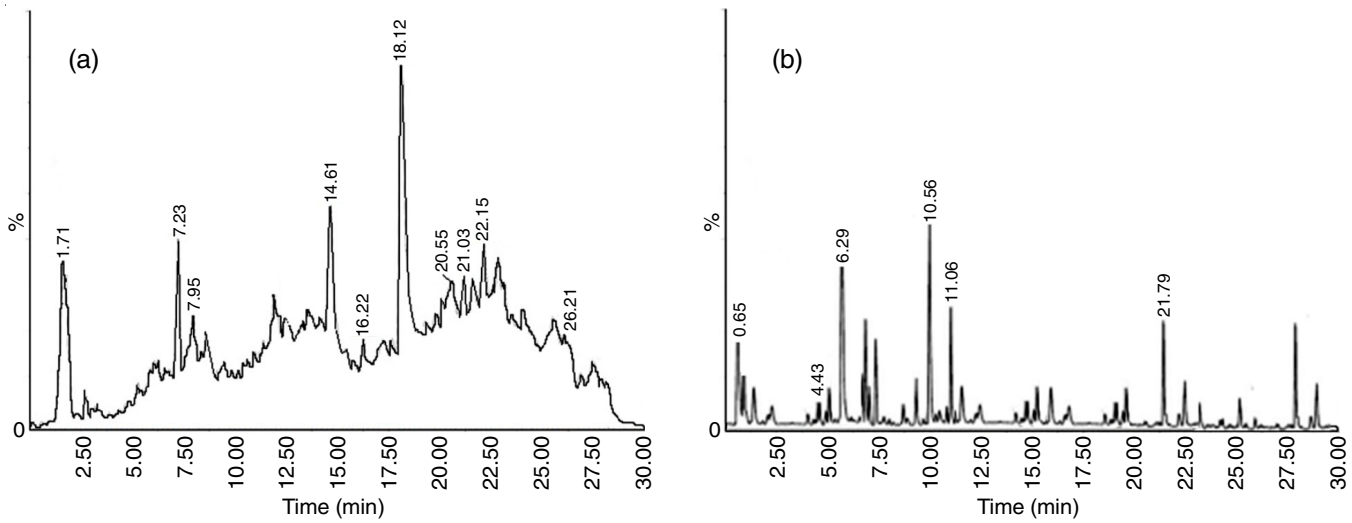

Fig. 1. LCMS study of (a) *Adiantum lunulatum* methanol leaves extract and (b) *Polystichum acrostichoides* methanol leaves extract

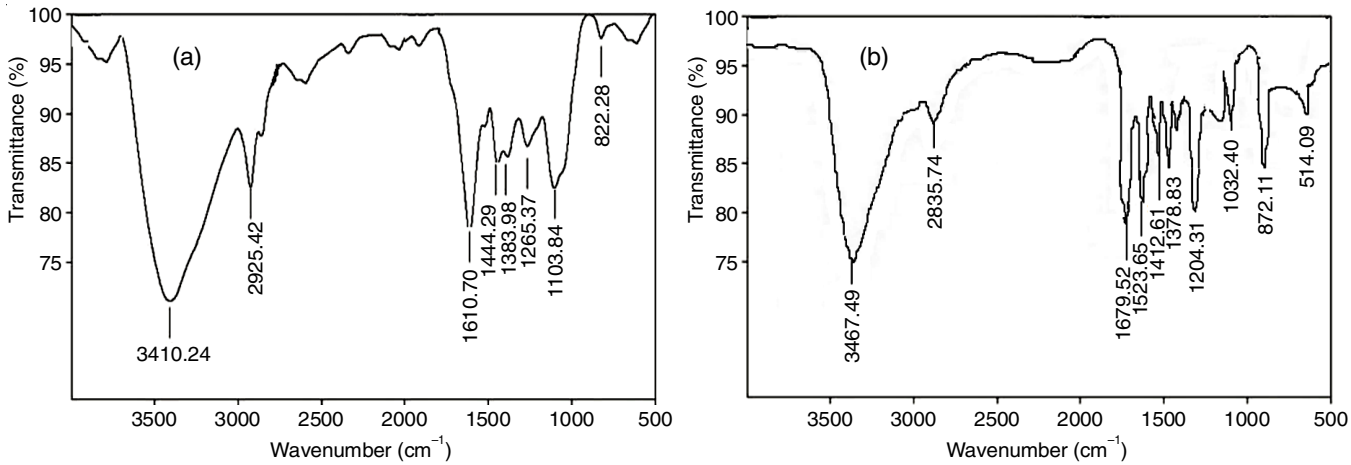

Fig. 2. IR spectra of (a) quercetin from *A. lunulatum* methanolic extract and (b) kaempferol from *P. acrostichoides* methanolic extract

extract (Fig. 2b) exihibits at peak at 3467.49 cm^{-1} as phenolic O-H *str.*, 2835.74 cm^{-1} as C-H *str.* and C=O *str.* at 1679.52 cm^{-1}. The absorption bands at 1573 and 1412 cm^{-1} denote the presence of aromatic ring, whereas 1204.31 cm^{-1} (C-O-C strand bend) of ketone and 872.11 cm^{-1} represented C-H bend of aromatic hydrocarbon.

NMR study: The ^{1}H NMR spectrum of isolated quercetin showed chemical shift values as follows: ^{1}H NMR (600 MHz) δ_H: 6.41 (1H, d, J = 2.4 Hz), 6.20 (1H, d, J = 2.4 Hz), 7.75 (1H, d, J = 3.0 Hz), 6.91 (1H, d, J = 9.6 Hz), 7.66–7.64 (1H, dd, J = 9.6, 3.0 Hz). In other hand, kaempferol revealed the chemical shift values (δ, ppm) as follows: 12.44 (s, 1H), 10.66 (s, 1H,), 10.04 (s, 1H), 9.24, 8.04 (d, J = 12.0 Hz, 2H), 6.92 (dt, J = 8.8 Hz, 2.4 Hz, 2H), 6.40–6.17 (dd, J = 8.8 Hz, 4 Hz, 2H), 6.30 (d, J = 2.1 Hz, 1H), 6.09 (d, J = 2.1 Hz, 1H).

^{13}C NMR spectra for quercetin was revealed as 148.34 (C-2), 137.52 (C-3), 177.64 (C-4), 162.79 (C-5), 99.59 (C-6), 165.96 (C-7), 94.75 (C-8), 158.54 (C-9), 104.80 (C-10), 124.44 (C-1′), 116.31 (C-2′), 146.53 (C-3′), 148.34 (C-4′), 116.53 (C-5′), 121.99 (C-6′). Whereas, kaempferol was identified as 177.73 (s, C-4), 165.97 (s, C-7), 162.30 (s, C-5), 161.27 (s, C-4), 157.43 (s, C-9), 148.36 (s, C-2), 136.01 (s, C-3), 130.98 (d, C-2, C-6), 123.45 (s, C-1), 116.79 (d, C-3, C-5), 105.78 (s, C-10), 98.42 (d, C-6), 92.49 (d,C-8).

Mass study: Fig. 3a-b depicted the mass spectra of the isolated quercetin and kaempferol. Fig. 3a depicted spectra of quercetin and resulted mass *m/z* value of 301.29 whereas, kaempferol resulted mass *m/z* value of 285.62 (Fig. 3b).

α-Amylase inhibition activity: Among both methanolic extracts, *A. lunulatum* showed comparatively better result than *P. acrostichoides*. Fig. 4 showed at various concentrations (50 to 800 µg/mL), *A. lunulatum* resulted percent inhibition 71.23 ± 0.97 at 800 µg/mL conc. with IC_{50} value of 419.39 ± 0.88 µg/mL. On other hand, *P. acrostichoides* extract showed percent inhibition 59.68 ± 0.93 at 800 µg/mL with IC_{50} value of 579.93 ± 1.55 µg/mL (Fig. 4b). The result was compared with standard acarbose which showed percent inhibition of 87.70 ± 0.16 at 800 µg/mL with IC_{50} value of 227.50 ± 0.14 µg/mL (Fig. 5).

α-Glucosidase inhibition activity: The same result followed for inhibition of α-glucosidase where *A. lunulatum* extract showed better result than *P. acrostichoides* when compared with the acarbose standard. Fig. 6 showed dose dependent increased percent inhibition with *A. lunulatum* extract (IC_{50} value: 526.12 ± 1.15 µg/mL) than *P. acrostichoides* extract (694.36 ± 2.08 µg/mL).

Glucose utilization in L-6 cells: *In vitro* study was done on L-6 cell lines glucose consumption and the results are depicted in Fig. 7. The results indicate that at 200 µg/mL dose,

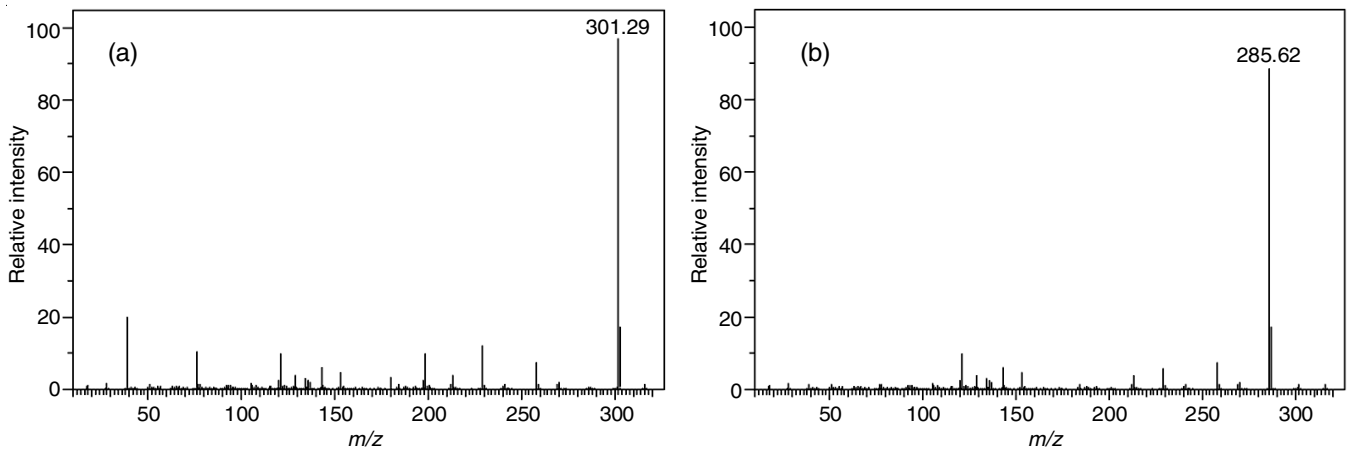

Fig. 3. Mass spectra of isolated (a) quercetin and (b) kaempferol

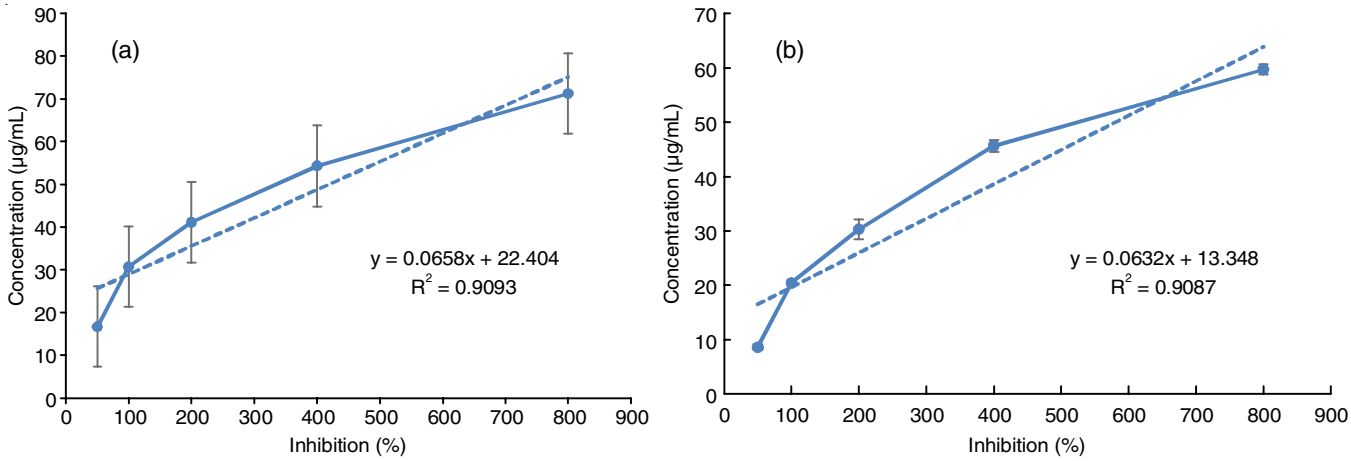

Fig. 4. α-Amylase inhibition activity of (a) methanolic extract of *A. lunulatum* and (b) methanolic extract of *P. acrostichoides*

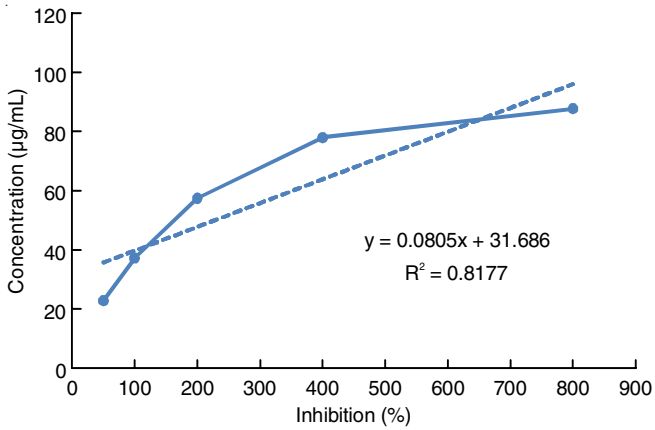

Fig. 5. α-Amylase inhibition activity of standard acarbose

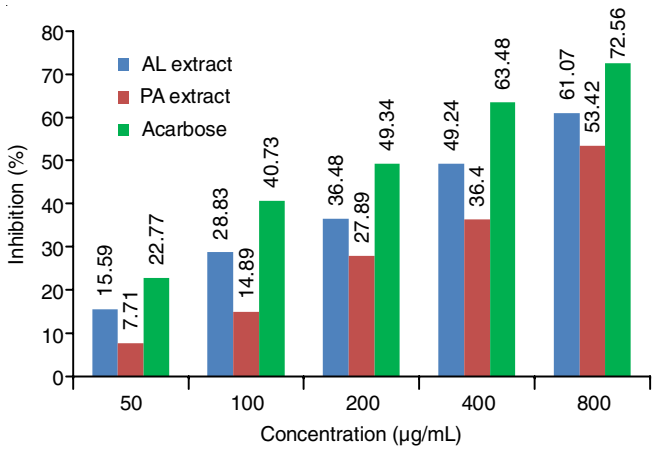

Fig. 6. α-Glucosidase inhibition activity of the methanolic extracts of *A. lunulatum* (AL) and *P. acrostichoides* (PA) with standard acarbose

the methanolic extract of *A. lunulatum* and *P. acrostichoides* enhanced the absorption of glucose by 45.24% and 32.11%, respectively as compared to the control. Insulin (an injectable medication) was used to compare the results of antidiabetic activity.

Fig. 7 showed *A. lunulatum* and *P. acrostichoides* extracts in combination with insulin not significantly increased the glucose uptakes or does not show any synergistic activity (132.97% and 130.14% for *A. lunulatum* and *P. acrostichoides*,

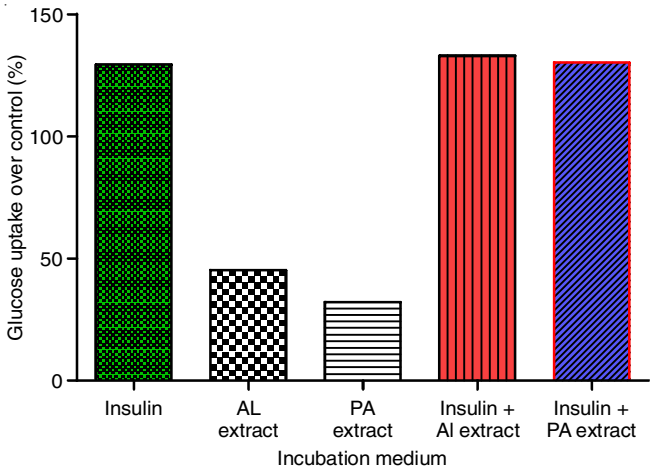

Fig. 7. Effect of the methanolic extracts of *A. lunulatum* (AL) and *P. acrostichoides* (PA) on glucose uptake in L6 cell line

respectively) where standard insulin uptakes glucose 129.28% but anyhow individual extracts revealed better activity where *A. lunulatum* extract uptakes more glucose than *P. acrostichoides*.

Conclusion

Using the L-6 cell line for glucose uptake experiments and α-glucosidase and α-amylase inhibition, the antidiabetic potential of *Adiantum lunulatum* and *Polystichum acrostichoides* extracts were assessed. There was a dose-dependent increase in the inhibitory effects of the methanolic extracts of *A. lunulatum* and *P. acrostichoides* on α-amylase and α-glucosidase due to presence of quercetin in *A. lunulatum* extract and kaempferol in *P. acrostichoides* plants as major bioactive compounds. Furthermore, percent glucose uptake was evaluated in L6 cell line and showed the maximum utilization of glucose uptake activity by *A. lunulatum* extract than *P. acrostichoides* extract. Anyhow, the overall results concluded that the methanolic extracts *A. lunulatum* and *P. acrostichoides* fern exhibit antidiabetic activity.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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