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# Extraction process optimization of flavonoid and *in vitro* amylase inhibitory effect of purified quercetin derivative from *Amorphophallus paeoniifolius* tubers

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*Amorphophallus paeoniifolius* (Elephant foot yam) is a prominent tuberous plant utilized across several parts of India to treat various ailments such as a tumour, haemorrhage, microbial infections, cough, bronchitis, diabetes, anaemia, and hepato-gastro and cardiovascular diseases. In this context, the present study aims to optimize the extraction process of the flavonoid and to study the *in vitro* amylase inhibitory effect of purified flavonoid moiety. The Shake flask method with different extraction solvents was adopted to quantify the flavonoid content. Central composite design (CCD) based response surface methodology (RSM) was formulated to optimize the extraction process. Three-dimensional preparative chromatography (3D PTLC) was executed to purify the flavonoid content and high-resolution liquid chromatography-mass spectrometry (HRLC-MS) was adopted to predict the structure. 3,5-dinitrosalicylic acid (DNS) based spectrophotometry method was used to determine the amylase inhibitory property. All the analyses were subjected to standard statistical tests. The developed model for the extraction optimization process was found to be near significant ($P = 0.242$) with temperature as a significant variable ($P = 0.029$), and a 107-fold increase (71.11±0.5 mg/g tissue) of flavonoid content was recorded. A strong yellow colour spot (flavonoid fraction) was eluted using 3D PTLC technique and the molecule was identified as quercetin derivative (*m/z* 447) by the direct MS method. Significant amylase inhibition (36.1±2.1%) recorded by purified quercetin derivative has documented the utilization of *A. paeoniifolius* tubers as classical traditional medicine.

**Keywords:** *Amorphophallus paeoniifolius*, Amylase inhibition, Quercetin derivative, Response surface methodology.

**IPC code; Int. cl. (2015.01)-** A61K 36/00, A61K 36/888, A61K 125/00, A61P 3/00

## Introduction

Type 2 diabetes mellitus (T2DM) is a chronic progressive metabolic disorder, probably the oldest one, and most prevalent disease among the human population. It is estimated that approximately 440 million people worldwide may be affected by T2DM by 2030[1]. According to the global scenario, India occupies first place in diabetes mellitus (DM) and it is expected that by 2030, around 80 million people may develop the disease[2]. The community studies conducted by Indian Medical Council Research (ICMR) have revealed that South India is more affected by DM than the Northern parts. Several factors such as smoking, alcoholism, physical inactivity, sedentary lifestyle, obesity, exposure to environmental toxins and genetic inheritance are responsible for the development of T2DM[3]. Similarly, T2DM can also co-occur along with gestational diabetes, cystic fibrosis, consumption of glucocorticoids, thiazide diuretics and statins, and the use of antiretroviral agents against AIDS. The progressive nature of T2DM may lead to risky complications such as retino, nephro, and neuropathies, foot ulcer, amputations, myocardial infarction, stroke, and premature death. To manage T2DM clinically, hypoglycemic drugs (oral/ subcutaneous etc.) like biguanide, dipeptidyl peptidase4 (DPP-IV) inhibitor, sodium-glucose cotransporter (SGLT2) inhibitor, insulin, GLP-1 agonists, sulfonylurea and thiazolidinedione are available in the market, but the adverse effects such as nausea, vomiting, allergy, pancreatitis, pernicious anaemia, respiratory tract infections and bladder cancer caused by these drugs should be considered as serious and life-threatening to human population[4]. Moreover, financial constraints in the procurement of allopathy drugs by poor and lower middle-class

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families are another great issue faced by many developing countries. Hence, the scientific community has started to practise traditional herbal-based medicine derived from plant sources to control T2DM. The main advantage is cost-effectiveness and fewer side effects, and several herbal formulations have been clinically documented its therapeutic potential against T2DM[5]. Recently, enzyme inhibitors derived from natural plant sources, especially against α-amylase has become popular and an attractive strategy to control T2DM. Plant species from families such as Rutaceae, Apocynaceae, Saxifragaceae, Zingiberaceae, Fabaceae, and Acanthaceae were proved to have significant α-amylase inhibitory activity[6].

*Amorphophallus paeoniifolius* (Family Araceae), also known as Elephant foot yam is a tropical tuber distributed in India, Philippines, Indonesia, Malaysia and Srilanka. In India, it is widely cultivated in Tamil Nadu, Punjab, Andra Pradesh, West Bengal, Kerala, Uttar Pradesh and Gujarat[7]. The tuber possesses numerous medicinal properties such as antitumour, anti-haemorrhage, antimicrobial, anti-inflammatory, antiviral, anticonvulsant, analgesic, antioxidant, antidiabetic, hepato, gastro, and cardioprotective, and control/ prevent anaemia, piles, constipation, anorexia, vomiting, cough, bronchitis, and dyspepsia[8-12].

Previous reports on the phytochemical screening of solvent extracts have revealed the presence of alkaloids, flavonoids, polyphenols, tannins, proteins, carbohydrates, sterols, flavones, saponins, fats and oils[13-15]. Significant reduction in blood and urine sugar levels and effective control over the glomerular filtration rate of acetone extract has been observed in streptozotocin-induced diabetic Wistar rats. The studies suspect the participation of polyphenols in the inhibition of α-amylase could be the probable reason for the antidiabetic property of *A. paeoniifolius*[16]. Similar documentation by Rahaman *et al.*[17] has revealed the potency of methanolic extract in a significant reduction of blood sugar level in streptozotocin-induced diabetic Albino mice and suggests the presence of lupeol may be responsible for the inhibition of α-amylase. However, none of the studies has attempted to compare and study the inhibitory effects between the extract and purified flavonoid fraction against α-amylase enzyme, and hence, the present study focussed on process optimization of extraction using experimental design, purification of flavonoid content, and its inhibitory effect against α-amylase enzyme.

## Materials and Methods

### Chemicals

Fungal amylase (S.D. Fine Ltd.), 3,5-dinitrosalicylic acid (S.d. Fine Ltd.), acarbose, quercetin (Merck), silica gel G (Merck). All other solvents used in the experimental analysis were analytical grade.

### Collection and preparation of plant material

The plant tubers were procured from a local market in Coimbatore, India, from September to December 2015. The plant sample was identified by P. Muthukumaran, Assistant Professor-II, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore. The tuber was washed with tap water to remove the mud and rinsed with distilled water. The outer peel was removed and the edible portion was cut into small pieces and subjected to the air shade drying process. The dried tuber was ground well in a pestle and mortar to a fine powder and stored at 4 °C for experimental analysis.

### Preparation of the extract

About 5 g of finely powdered tuber sample was dissolved in 50 mL of solvents (methanol, ethanol, acetone, petroleum ether, hexane, distilled water, and ethyl acetate) and kept in an orbital shaker for 12-16 h (Shake flask method). The obtained residue was re-extracted under the same condition. The extracts were filtered with Whatman number no. 1 filter paper and the filtrate was used for estimation of total flavonoid content[18]. The excess filtrate was stored at 4 °C for further studies.

### Estimation of total flavonoid content (TFC)

To 0.1 mL of the extract, distilled water was added to make up the volume to 5 mL. To this, added 0.3 mL of 5% $NaNO_2$. After 5 minutes, added 3 mL of 10% $AlCl_3$ and mixed well. Six minutes later, 2 mL of 1M NaOH was added and the absorbance was recorded spectrophotometrically at 510 nm. Quercetin was used to construct the standard calibration curve[18].

### CCD based RSM design for optimization of the flavonoid extraction process

Optimization of extraction of flavonoid content was done using CCD based RSM experimental design (eight cube points, six axial points, and six centre points in cube). Factors such as temperature (°C), time duration (h) and material ratio (g/mL [tissue: solvent]) with three different levels were selected for the design (total of 20 experimental runs that

constitute six dummy variables) (Table 1). A second-order polynomial equation was used to express the developed experimental model with the response (y) [flavonoid content (mg/g tissue)] as a function of the coded independent variables and $x_1$ (temperature), $x_2$ (time duration) and $x_3$ (material ratio) were the independent variables that affect the responses. The equation is predicted as follows:

$$y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{i=1}^{n} a_{ij} x_i x_{j\, i<j}$$

where, $a_0$, $a_i$ (i= 1, 2 . . . k), $a_{ii}$ (i = 1, 2 . . . k) and $a_{ij}$ (i = 1, 2 . . . k; j = 1, 2 . . . k) were regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and 'k' is the number of variables. One way ANOVA (5% level of significance) was adopted to analyse the validity of the experimental model and to identify the significant factors that affect the extraction process. Surface, contour, and interaction plots were predicted to analyse the response/ optimal condition of the selected factors.

**Purification of flavonoid by 3D preparative thin-layer chromatography (PTLC)**

The glass plates (20 cm ×20 cm) were coated with silica gel G (2 mm thick) and the plates were dried at room temperature. The plates were then activated at 100 °C for 30 minutes in an oven, brought to room temperature and spotted 500 µL of the optimal extract from 2 cm above the edge of the plate. The mobile phases adopted as first dimension, second dimension and third dimension were toluene: chloroform: acetone (40:25:35), chloroform: methanol: formic acid (85:15:1) and ethyl acetate: ethanol: water (5: 1: 5), respectively.

Table 1 — Real values of three variables with three different levels adopted in full factorial CCD based RSM design

| Run order | Temperature (°C) | Time duration (h) | Material ratio (g/mL) |
|---|---|---|---|
| 1 | 50 | 1.5 | 20 |
| 2 | 55 | 1.84 | 15 |
| 3 | 55 | 1 | 6.59 |
| 4 | 55 | 0.16 | 15 |
| 5 | 60 | 0.5 | 10 |
| 6 | 55 | 1 | 15 |
| 7 | 50 | 0.5 | 20 |
| 8 | 46.59 | 1 | 15 |
| 9 | 55 | 1 | 15 |
| 10 | 60 | 1.5 | 20 |
| 11 | 50 | 1.5 | 10 |
| 12 | 63.41 | 1 | 15 |
| 13 | 60 | 0.5 | 20 |
| 14 | 55 | 1 | 15 |
| 15 | 50 | 0.5 | 10 |
| 16 | 55 | 1 | 15 |
| 17 | 55 | 1 | 23.41 |
| 18 | 60 | 1.5 | 10 |
| 19 | 55 | 1 | 15 |
| 20 | 55 | 1 | 15 |

The developed chromatogram was visualized under far UV light to identify the presence of flavonoid and phenolic acids. The identified flavonoid spots were carefully eluted, dissolved in phosphate buffer (pH 7.0), and centrifuged at 5,000 rpm for 5 minutes. The supernatant was collected, lyophilized, and stored at 4 °C for further use[18,19].

**High-resolution liquid chromatogram mass spectrometry (HRLC-MS)**

The PTLC eluate was subjected to direct HRLC-MS (electron spray ionization (ESI) in both positive and negative mode) analysis as per the slightly modified procedure proposed by Sathishkumar *et al*[18]. The analysis was carried out in 1260 infinity Nano HPLC with Chipcube of Agilent tech (USA). The mobile phase was composed of 0.3% formic acid (pH 2.2) [phase A] and acetonitrile (phase B). Gradient elution was performed at 0.9 mL/min as follows: i) phase A: 88% (70 min); ii) phase B: 9–15% (0–21min), 15–22% (21–45min), 22–35% (45–60min), 35–90% (60–65 min) and 90% (65–70 min). The eluate was monitored by PDA (multi-wavelength) detector at 260 nm. The mass spectra was scanned in the range 100-1000 amu from 0 - 0.39 mins to obtain direct peaks.

***In vitro* amylase inhibition assay**

A modified method of Bernfeld proposed by Hema *et al.*[20] was adopted to investigate the *in vitro* α-amylase inhibitory activity. About 0.1 mL of the sample was added in the Blank and Test tubes, and 0.1 mL of amylase enzyme in the Test and Control tubes, respectively. Added 1 mL of 50 mM phosphate buffer (pH 7.0) and 0.5 mL of 1% starch into all the tubes and incubated at 37 °C for 10 minutes. Added 1 mL of 3,5- DNS reagent into all the tubes, mixed well, and incubated in a boiling water bath for 10 minutes. Cooled the tubes and read the absorbance at 540 nm against blank. Acarbose was used as the positive control to compare with the test sample. The maltose liberated was determined against a constructed standard maltose curve. The enzyme activity and inhibitory activity was calculated using the following formula:

Enzyme activity (U/ mL) = [(Cm × Ez) / (Mw × It)] × dilution factor

where Cm is Conc. of maltose liberated, Ez is the enzyme used (in mL), Mw is Mol wt. of maltose, and It is incubation time (min).

Enzyme inhibition (%) = [(Absorbance of control – Absorbance of test) / Absorbance of control] × 100

**Pairwise and multiple sequence analysis (Dry lab bioinformatics approach)**

Online bioinformatics tools such as EMBOSS Water and EMBOSS Dotmatcher were used to study pairwise sequence analysis. EMBOSS Clustal Omega was adopted to perform and analyse multiple sequence analysis.

**Statistical analysis**

The values depicted in the results were expressed as mean±S.D. Karl Pearson correlation coefficient and simple regression equation values were calculated using MS excel 2010 version. Design Expert 11 trial version was used for CCD based RSM design and one way ANOVA.

## Results and Discussion

**Quantification of TFC in tuber extracts**

The total flavonoid content was estimated in different solvent extracts through the shake flask method and results were expressed as quercetin equivalents (Fig. 1). The investigation has proved methanol as an optimal solvent with a yield of about 0.66±0.01 mg/g tissue. The descending order of solvents according to the TFC yield (mg/ g tissue) was as follows: Methanol (0.66±0.01) > ethanol (0.45±0.03) > distilled water (0.38±0.02) > acetone and hexane (0.31±0.02) > ethyl acetate and petroleum acetate (0.09±0.03).

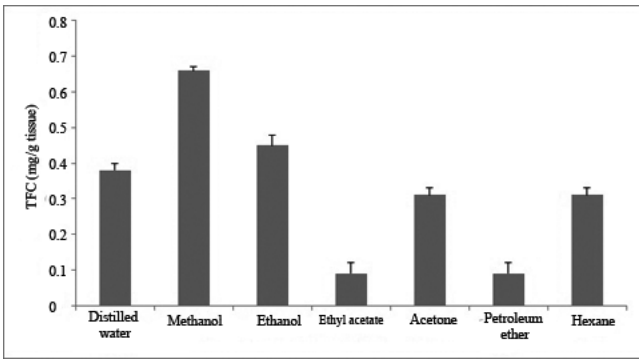


Fig. 1 — Effect of different solvents in the extraction of TFC by shake flask method.

Flavonoids are prominent natural products of plants, a class of secondary metabolites that possess polyphenolic structures and are widely distributed in fruits, leaves, root, and stem. They are vital components in cosmetic, pharmaceutical and nutraceutical applications. Flavonoids possess diversified biological activities such as UV filter, hepato, reno, and cardioprotective effects, hormone mimic effect, enzyme inhibitory activities, antimicrobial, antioxidant, antidiabetic, antiviral, anticholinesterase, antihypercholesterolemic, antimutagenic, anticarcinogenic, antiplatelet, antimalarial, and antiinflammatory activities[21]. Hence, exploring the presence of existing and new flavonoid molecules in plant species has received much attention from the scientific community.

In the current investigation, alcoholic solvents (methanol and ethanol) have proved to be best in the extraction of flavonoid content from the tubers of *A. paeoniifolius*. Methanol and ethanol were considered as best, and distilled water, acetone, and hexane as moderate, and ethyl acetate and petroleum ether were poor solvents in the extraction of TFC. The extraction efficacy of phytochemicals depends on variables such as particle size, structural variations, pH, sample volume, extraction method, type of solvent and temperature[22]. Particularly, the selection of solvent with optimal polarity to extract both the polar and nonpolar flavonoid molecules is always a challenging task. Generally, alcoholic solvents play a vital role in the extraction of flavonoid molecules because of their bipolar nature. The aromatic ring can form weak hydrophobic interaction with nonpolar flavonoid and –OH group can form a hydrogen bond with polar flavonoid, and both types of molecules can be effectively extracted from the raw material[23]. Previous reports by Nataraj *et al.* [24] and Peraman *et al.*[25] have documented the appreciable quantity of flavonoid (46.33 mg/ g) and (107 mg/g) in the methanolic tuber extracts obtained through exhaustive soxhlet method (approx. 20 cycles) at high temperature and more consumption of solvents. The current study has proved the coherence in the selection of solvent with less consumption and minimal temperature (room temperature), but TFC obtained was very poor.

**CCD based RSM of extraction process optimization of TFC**

In the current investigation, CCD based RSM experimental design was formulated to understand and study the significance of three different variables viz., temperature (°C), time duration (h) and material ratio

(raw material/ methanol [g/ mL]) in the extraction of TFC. A suitable empirical model was developed to record and validate the involvement/ interactions between the variables responsible for TFC extraction. The optimal condition of variables for the extraction process was found to be 60 °C, 1.5 h and 1:20 (Table 2), which was endorsed by the surface and contour plots (Fig. 2). The results have recorded the range of TFC. It was observed that the CCD based RSM experimental design has improved the flavonoid content (107 fold increase). One way ANOVA of the quadratic and cubic model was found to be near significant (p = 0.242) and significant (p = 0.0008), respectively, which, proved the efficacy and validity of the selected variables and model of the extraction process. Under quadratic model, temperature was found to be significant at 5% level (p = 0.029), whereas, in cubic model, time duration (p = 0.0057) and material ratio (p = 0.0172) were found to be significant in the extraction of TFC. The results also proved that the linear effect of a variable (p = 0.104) is more influencing than quadratic (p = 0.493) and interactive (p = 0.356) effects in the extraction process (Table 3). The second-order polynomial equation generated in terms of multiple regression equation has expressed very well about the potency of the process towards the extraction of flavonoid content.

$$Y = 448 - 9.5\,x_1 - 115\,x_2 - 21.3\,x_3 + 0.052\,x_1^2 + 16.1\,x_2^2 + 0.153\,x_3^2 + 1.07\,x_1 x_2 + 0.294\,x_1 x_3 + 1.34\,x_2 x_3$$

Where Y represents the TFC and $x_1$, $x_2$, and $x_3$ represents the independent variables selected for the extraction process design. The negative co-efficients ($x_1$, $x_2$ and $x_3$) revealed the inverse relationship of variables with the response (TFC), and the positive co-efficients ($x_1^2$, $x_2^2$, $x_3^2$, $x_1 x_2$, $x_1 x_3$, and $x_2 x_3$) showed the direct relationship of variables with the response. The main (independent) and interactive effects of variables were depicted in Fig. 3.

To improve the yield of flavonoid content, CCD based RSM design analysis was extended further to find an optimal condition with the selected variables viz., temperature (°C), time duration (hrs) and material ratio (g/ mL). This is a powerful statistical technique adopted by several industries to optimize an experimental setup for cost-effectiveness and reduced time consumption[26]. Previous reports have recorded the impact of a few variables such as temperature, pH, material ratio, extraction time duration and modifiers in the extraction process of polyphenols and flavonoids from plant species[27-29]. In the present study, a full factorial CCD model was used to correlate the selected variables with the response by generating a quadratic based polynomial equation. Surface, contour, main, and interactive plots were used to support the results. According to quadratic model, the variables were ranked in the descending order based upon p-values: Temperature (p = 0.029) > material ratio (p = 0.283) > time duration (p = 0.595).

Table 2 — Optimization of extraction of total flavonoid content recorded by CCD based RSM

| Run order | Temperature (°C) | Time duration (h) | Material ratio (g/mL) | Flavonoid content (mg/g tissue) |
|---|---|---|---|---|
| 1 | 50 | 1.5 | 20 | 7.62 ± 0.32 |
| 2 | 55 | 1.84 | 15 | 19.24 ± 0.51 |
| 3 | 55 | 1 | 6.59 | 21.20 ± 1.2 |
| 4 | 55 | 0.16 | 15 | 42.03 ± 2.3 |
| 5 | 60 | 0.5 | 10 | 42.07 ± 0.5 |
| 6 | 55 | 1 | 15 | 26.29 ± 0.5 |
| 7 | 50 | 0.5 | 20 | 14.98 ± 1.6 |
| 8 | 46.59 | 1 | 15 | 25.21 ± 1.2 |
| 9 | 55 | 1 | 15 | 20.38 ± 2.8 |
| 10 | **60** | **1.5** | **20** | **71.11 ± 1.5** |
| 11 | 50 | 1.5 | 10 | 21.71 ± 0.9 |
| 12 | 63.41 | 1 | 15 | 20.63 ± 1.0 |
| 13 | 60 | 0.5 | 20 | 44.02 ± 2.1 |
| 14 | 55 | 1 | 15 | 19.30 ± 0.3 |
| 15 | 50 | 0.5 | 10 | 18.71 ± 0.8 |
| 16 | 55 | 1 | 15 | 19.75 ± 0.2 |
| 17 | 55 | 1 | 23.41 | 38.94 ± 1.5 |
| 18 | 60 | 1.5 | 10 | 32.08 ± 0.7 |
| 19 | 55 | 1 | 15 | 23.94 ± 1.7 |
| 20 | 55 | 1 | 15 | 17.33 ± 1.2 |

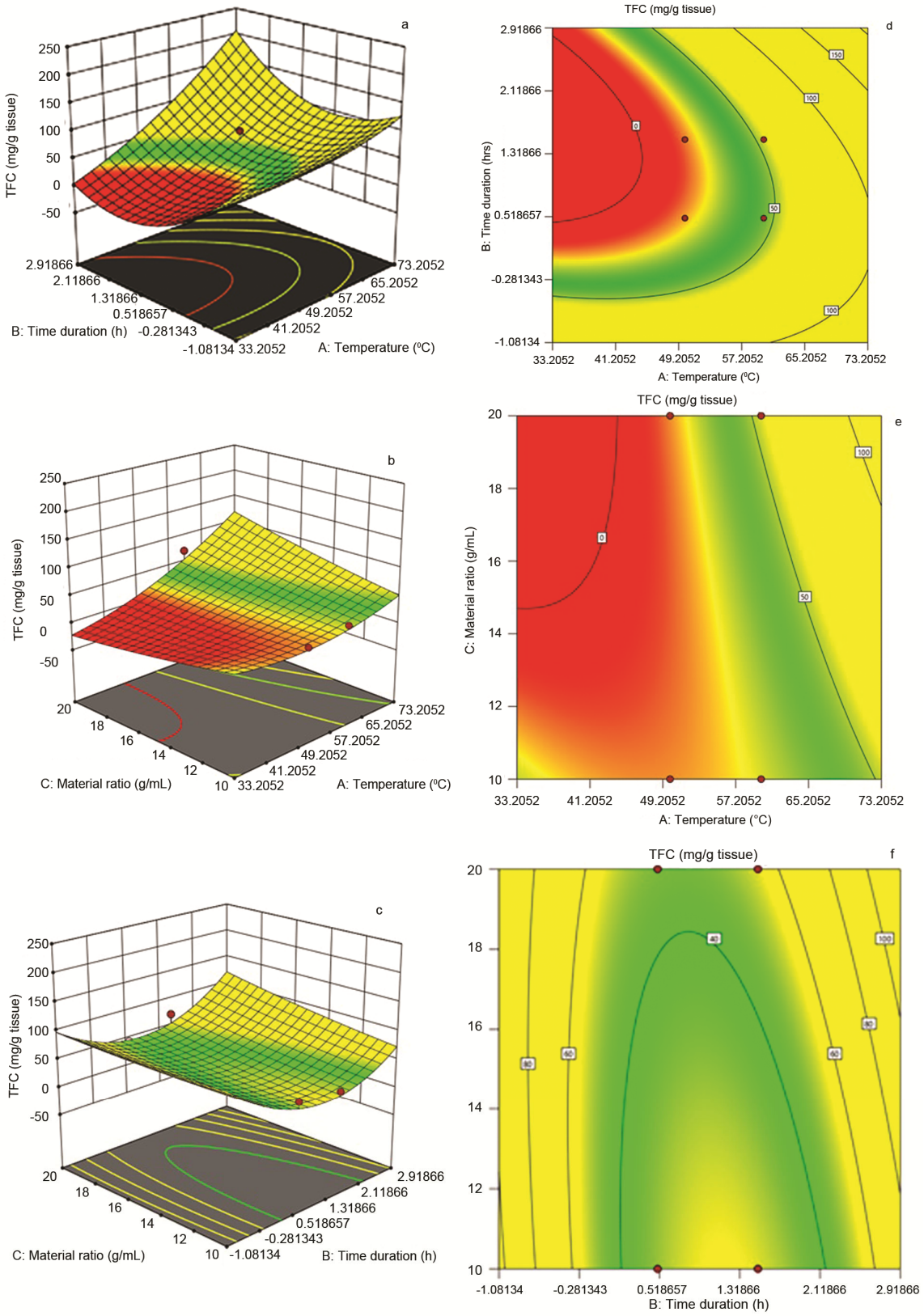


Fig. 2 — Impact of the variables in full factorial CCD based RSM extraction process of flavonoids (a, b & c): surface plot; (d, e & f): Contour plot.

Table 3 — One way ANOVA of quadratic model derived from CCD based RSM experimental design

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Model | 9 | 2273.68 | 252.63 | 1.58 | 0.242 |
| Linear | 3 | 1283.08 | 427.69 | 2.68 | 0.104 |
| Temperature (°C) | **1** | **1029.55** | **1029.55** | **6.46** | **0.029** |
| Time duration (h) | 1 | 47.96 | 47.96 | 0.30 | 0.595 |
| Material ratio (g/mL) | 1 | 205.56 | 205.56 | 1.29 | 0.283 |
| Square | 3 | 411.78 | 137.26 | 0.86 | 0.493 |
| Temperature (°C)*Temperature (°C) | 1 | 24.67 | 24.67 | 0.15 | 0.702 |
| Time duration (h)*Time duration (h) | 1 | 234.72 | 234.72 | 1.47 | 0.253 |
| Material ratio (g/ mL)*Material ratio (g/mL) | 1 | 212.13 | 212.13 | 1.33 | 0.276 |
| 2-Way interaction | 3 | 578.83 | 192.94 | 1.21 | 0.356 |
| Temperature (°C)*Time duration (h) | 1 | 57.57 | 57.57 | 0.36 | 0.561 |
| Temperature (°C)*Material ratio (g/mL) | 1 | 432.09 | 432.09 | 2.71 | 0.131 |
| Time duration (h)*Material ratio (g/mL) | 1 | 89.17 | 89.17 | 0.56 | 0.472 |
| Error | 10 | 1594.60 | 159.46 | | |
| Lack-of-Fit | 5 | 1539.92 | 307.98 | 28.16 | 0.001 |
| Pure error | 5 | 54.69 | 10.94 | | |
| Total | 19 | 3868.28 | | | |

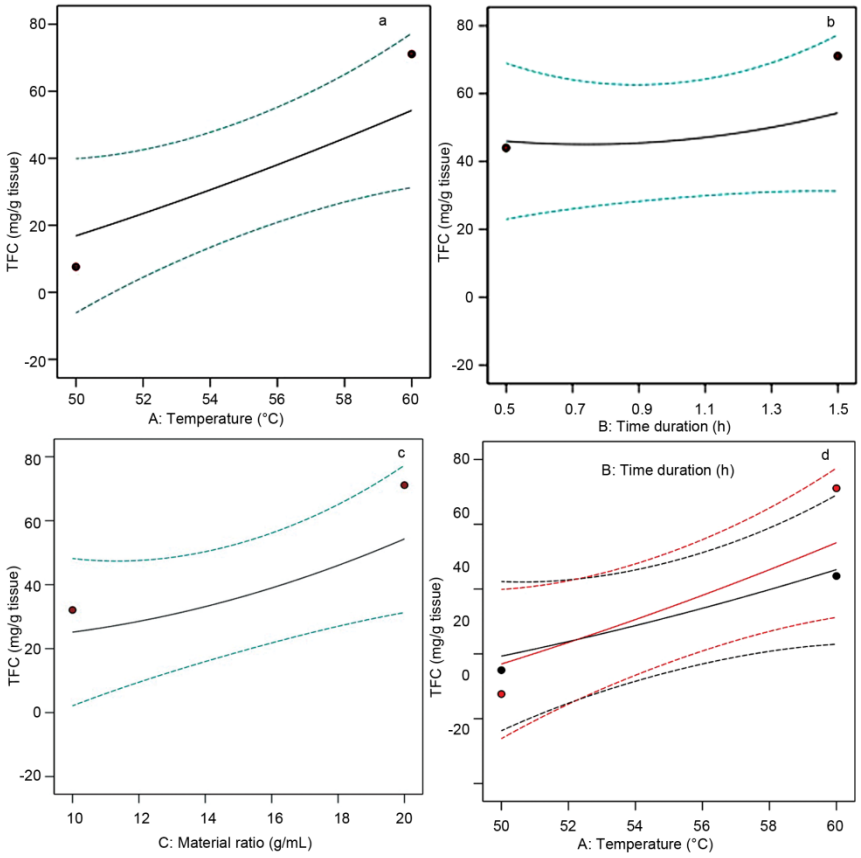


Fig. 3 — Main (a, b & c) and interactive (d) effects of the variables in the optimization of flavonoid extraction.

The surface and contour plots indicated in Fig. 2a & d possess an intense red colour area downwards the y-axis (i.e., towards time duration) which has recorded the lowest flavonoid yield. Similarly, the graph depicted in Fig. 3b (black colour dots) and Fig. 3d (black and red colour lines indicates the time duration at 0.5 and 1.5 h, respectively) clearly showed a near-constant effect of time duration against the flavonoid yield (mg/ g tissue) proving its insignificancy in the process ($p = 0.595$). An

experimental documentation of run order no. 13 (44.02±2.1) and no. 10 (71.11±1.5), respectively, has justified the above statement. Increased time duration during the initial period has improved the flavonoid yield, but prolonged time has a negative effect/ decrement in the leaching improvement of the flavonoid content. The probable reason for this decrement in the yield of flavonoid content was due to the attainment of equilibrium of extraction process which follows Fick's second law of diffusion and oxidation-based degradation of flavonoid molecules[27,30]. In contradiction, the present study has recorded a slight decrement in the flavonoid yield in the initial period (0.5–0.7 h) and a near steady improvement in the extraction of TFC over another phase of the period (0.7–1.5 h).

An exclusive increase in temperature has improved the TFC (mg/ g tissue) to a greater extent which was revealed by a strong yellow colour upward in the x-axis in both the surface and contour plots (Fig. 2a & d; b & e). The experimental record of run order no. 1 (7.62±0.32) and no. 10 (71.11±1.5) has evidenced the significant effect of temperature in the extraction process. A gradual increase in temperature with every 8 ºC rise has drastically increased the yield of flavonoid content (Fig. 3a & d). Increased thermal energy may act as a physical catalyst (thermal decomposition) that disrupts the cell wall mediated covalent interactions and alter the non-covalent bondings which destabilize the membrane fluidity, and leach more phytochemicals, including flavonoids out of the cell. Normally, higher temperatures may have a negative impact against the response, i.e., decomposition of compounds through redox and hydrolysis reactions, and aggregation of monomers to polymers by polymerization reaction[31]. But our current investigation doesn't record any such negative effect, instead a significant improvement ($p = 0.029$) in the extraction of flavonoid content was noticed throughout the temperature range.

The impact of material ratio was explained in Fig. 2c & f. The intense yellow colour observed throughout the y-axis in surface and contour plots revealed the significance of material ratio in leaching the flavonoids from the plant tissues. Exclusively, alcohols promote the membrane fluidity destabilization of plant cells by disrupting the hydrogen bonds between the polar heads of lipid bilayers and enhancing their permeability inside the cell. The penetrated alcohols can interact with the phytochemicals and drastically leach them outside the cell. This mass transfer effect was due to the existence of a larger concentration gradient created due to the high material ratio, which significantly dissolute more phytochemicals from the sample matrix[32]. A similar positive effect of material ratio, as evident from Fig. 3c, was observed in our present study. A significant ($p = 0.283$) leaching effect of flavonoid content over the range of material ratio (1:10 to 1:20) was observed throughout the study.

**Purification of flavonoid by 3D PTLC and structural prediction using HRLC-MS analysis**

The optimal methanolic extract obtained from CCD based RSM design was subjected for purification of flavonoid using 3D PTLC technique. A strong yellow colour single spot flavonoid moiety visualized under far UV light after the three-dimension purification process was eluted (Fig. 4) and subjected for

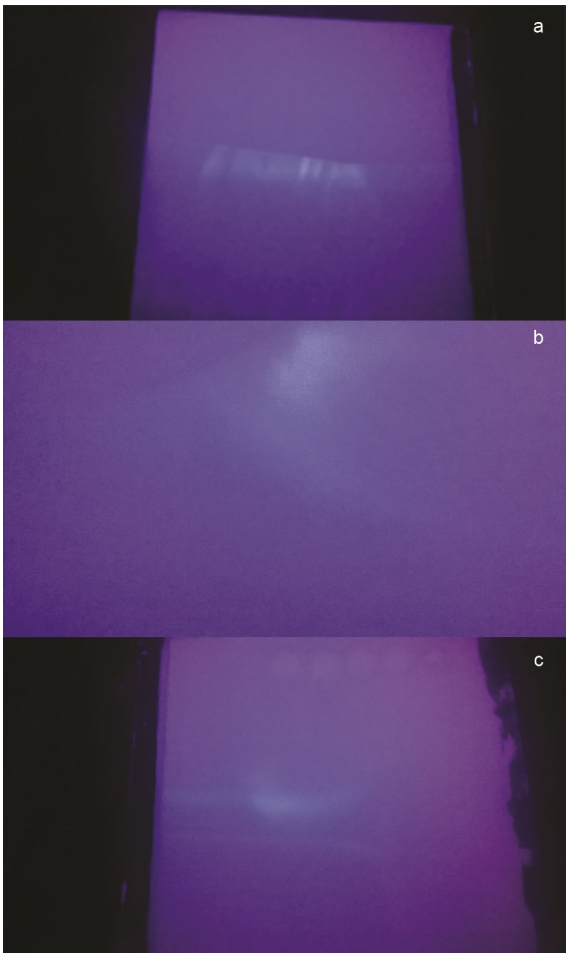


Fig. 4 — Purification of yellow colour flavonoid using 3D PTLC and visualization under far UV light, a) visualization of flavonoid in one dimension version, b) two-dimension version, c) three dimension version.

structural prediction using HRLC-MS analysis. A single peak recorded at m/z 447 (Rt = 0.074) revealed the presence of quercetin derivative (Fig. 5).

TLC is a simple, versatile, cost-effective and user-friendly technique for the identification of natural compounds. But the greatest disadvantage is layer thickness, temperature, sample size, and dampness of TLC plate, which can cause a peak broadening, and thereby, an expanded $R_f$ value[33]. Moreover, conventional one-dimensional TLC/PTLC is not efficient to separate and purify a more complex sample matrix. In that case, a multidimensional TLC/PTLC would be the correct choice which can adopt two different mobile phases or bilayer stationary phase for purification of natural compounds[34]. The main advantage of such multidimensional planar chromatography is increased resolving capacity and thereby, reduction in the peak broadening of a purified compound. In this context, a new and novel 3D PTLC was executed to purify the flavonoid compound. The optimal extract (60 °C, 1.5 h and 1:20) obtained through CCD based RSM design was used for the above-said purpose. A single strong yellow colour spot observed under far UV light has revealed the presence of flavonoids. The distribution surface area of flavonoid was greater under a single dimension (Fig. 4a) and gradually decreased in further dimensions to form a narrow surface area (Fig. 4b & c) which is easier to elute the targeted compound. Very limited attempts have been made to identify and purify the flavonoid and other phenolics from *A. paeoniifolius* tubers. An earlier report has documented the purified flavonoid such as quercetin and gallic acid in the alcoholic tuber extracts[8]. The present attempt in the purification of flavonoid was successful and able to elute a single spot from 3D PTLC technique.

The purified PTLC eluate was subjected to direct ESI/ MS analysis. A single peak recorded with *m/z* 447 has revealed the presence of quercetin derivative. Previous reports by Saldanha *et al.*[35], [*m/z* 447] and Diaconeasa *et al.*[36] [*m/z* 449] on the record of quercetin derivative has supported the current result and this study is the first to report the purified quercetin derivative in the tubers of *A. paeoniifolius.*

**Comparative analysis of *in vitro* amylase inhibitory effect**

Acarbose, methanolic crude extract, and quercetin derivative were compared and recorded for amylase inhibitory effect. A simple regression equation (y = 0.0022x) obtained from the standard calibration of the maltose curve was used to calculate the concentration of liberated maltose (µg) which, was adopted to calculate amylase activity (U/mL). A strong correlation coefficient ($R^2$ = 0.9329) between the concentration of maltose and absorbance was noticed. The control amylase activity was found to be 0.01±0.002 U/mL and used to calculate the inhibitory activity of test samples. The positive control acarbose revealed a significant inhibition (77.33±1.7%) against amylase. The crude methanolic extract recorded a poor amylase inhibitory activity (18.18±2.5%) than the purified quercetin derivative (36.1±0.5%) which proved the potency of the quercetin derivative as a promising hypoglycaemic agent. The comparative inhibitory effect was depicted in Fig. 6.

The comparative result of *in vitro* amylase inhibitory effect of various molecular entities (100 µg/mL) has been arranged in ascending order: methanolic extract (18.18±2.5%) <quercetin derivative (36.1±0.5%) < acarbose (77.33±1.7%). Compared to crude extract, purified quercetin derivative has recorded a 1.99-fold increase in the

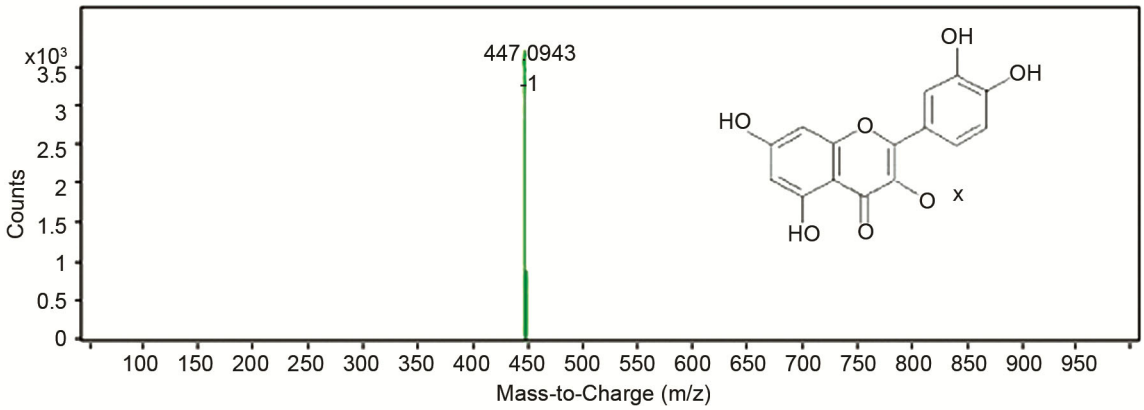


Fig. 5 — Structural prediction of quercetin derivative using direct MS method.

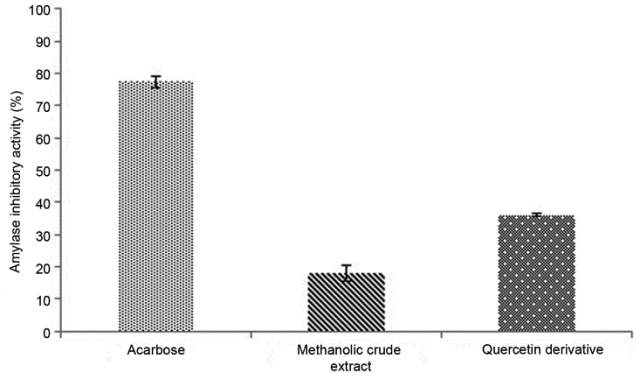


Fig. 6 — Comparative amylase inhibitory effect between acarbose, methanolic crude extract and quercetin derivative.

inhibitory effect against amylase enzyme. The positive control acarbose has recorded a 4.24-fold increase and a 2.14-fold increase of amylase inhibitory effects than the extract and quercetin derivative, respectively. Even though acarbose exhibited very high inhibitory activity, it may not be preferred to treat T2DM because of its severe gastrointestinal adverse effects. Hence, natural flavonoids are selected to restrain postprandial hyperglycemia because of their unharmfulness towards GI tract[37]. An effective glycemic index (lesser than 6.5% HbA1C) is mandatory to regulate T2DM and thereby, irreversible microvascular complications leading to several cardiovascular diseases can be prevented. Pharmacotherapy promises either an effective control or slow / much-delayed progression of T2DM. The currently available drugs can control T2DM by adopting strategies such as increased uptake of hepatic glucose by activated protein kinase and thereby, decreased gluconeogenesis (biguanide derivatives), activation of glucagon like peptide-1 (GLP-1) receptor to stimulate insulin secretion (Liraglutide), inhibition of glucose reabsorption in proximal convoluted tubule by the sodium–glucose cotransporters (SGLT2 inhibitors) (canagliflozin), increased insulin secretion of the pancreas by blocking potassium-ATP channels (Sulfonylureas), insulin therapy, alpha-glucosidase inhibitors (voglibose and acarbose), and bile acid sequestrants like colesevelam[4].

The main carbohydrate source after hydrolysis of starch or glycogen during the digestion process is a mixture of oligosaccharides and maltose, and the biochemical reaction is catalysed by isoforms of amylase (salivary and pancreatic). The formed disaccharide is further catalysed majorly by maltase-glucoamylase or minorly by sucrose-isomaltase to glucose which is readily absorbed into the bloodstream and effectively distributed to different systemic tissues. The first line digestive, carbohydrase is amylase and major activity (approx. 70%) is mediated by pancreatic type rather than salivary one. The catalytic triad of amylase is in α/β barrel and composed of Asp197, Glu223 and Asp300[38]. It is reported that flavonoid glycosides are stronger inhibitors of amylase than flavonols. Hence, quercetin derivatives such as rutin, quercetin-arabinoside, quercetin-glucoside and quercetin-xyloside possess strong amylase inhibitory effect than flavonols such as quercetin. An increase in the sugar moiety may increase the polarity of a flavonoid molecule and thereby, enhance the formation of hydrogen bond with the amylase, preventing the access of the substrates (starch or glycogen or oligosaccgarides) towards the substrate-binding/ catalytic sites[39]. Similarly, other types of non-covalent interactions such as van der Waals forces and hydrophobic interactions also play a vital role in the inhibition of amylase. A near similar mechanism is also expected between acarbose and quercetin derivatives[40].

Previous docking studies on porcine and human pancreatic α-amylase with natural low molecular weight flavonoid inhibitors such as quercetin, quercetin hexosides and other lead molecules have shown strong inhibitory activity[40,41]. Multiple sequence analysis (msa) using CLUSTAL OMEGA online tool between *A. nidulans (*AAF17103.1), and human (AAA51724.1) and porcine (pdb|1KXQ|D) pancreatic amylases has proved the presence of catalytic important consensus amino acids such as aspartate (198), glutamate/ asparate (234), isoleucine/ valine (235), glutamate (301) and alanine (306) (Fig. 7). The optimal local alignment (EMBOSS WATER) results have revealed a significant similarity existing between the *A. nidulans* and human pancreatic amylase (60.8%), and porcine pancreatic amylase (60.4%). The dot plot analysis (EMBOSS DOTMATCHER) between *A. nidulans* and human pancreatic amylase (W=75 & T =25), and *A. nidulans* and porcine pancreatic amylase (W=75 & T =15) has proved the existence of strong similarity of amino acid strings between the location 50 – 250 (Fig. 8). The overall dry lab bioinformatics approach has proved the consensus between *A. nidulans*, human and porcine pancreatic amylases, and also justified the selection of fungal amylase in the current study.

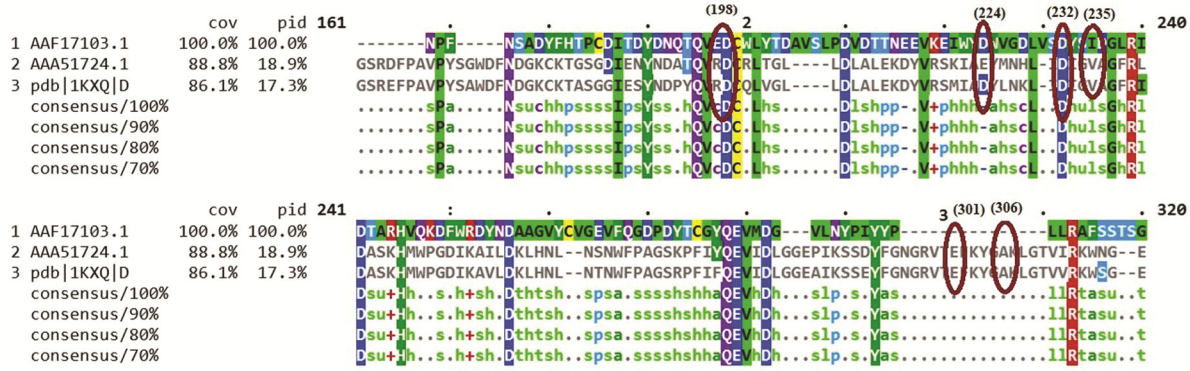


Fig. 7 — Multiple sequence analysis revealing the consensus catalytic amino acids between *A. nidulans*, human and porcine pancreatic α-amylases.

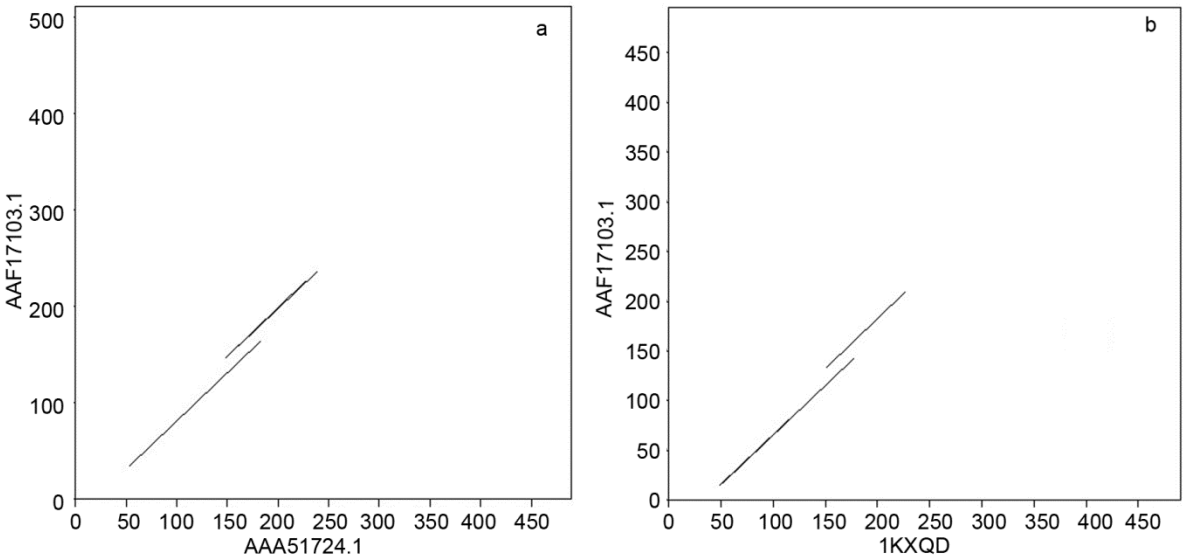


Fig. 8 — Dot plot analysis between the selected α-amylases. a) *A. nidulans* and human pancreatic α-amylase, b) *A. nidulans* and porcine pancreatic α-amylase.

An *in vivo* study performed by Arva *et al.*[16] has strongly established the antidiabetic effect in streptozotocin-induced male Wistar rats. About 37% reduction of fasting blood sugar and 41% reduction of glomerular filtration rate (GFR) was established by the organic solvents tuber extract. A similar correlated result has been recorded by Rahaman *et al.*[17] in Swiss Albino male mice. But, nobody has attempted to study the amylase inhibitory activity of *A. paeoniifolius* extract and purified flavonoid moiety. The present study has recorded the potency of purified quercetin derivative in inhibiting amylase enzyme.

To summarize, even though the conventional, universally accepted shake flask method has recorded low flavonoid yield, a drastic improvement in the content was noticed by the execution of CCD based RSM design. The RSM was validated by quadratic (near significant to 5% level) and cubic (significant at 0.1% level) models with identifying temperature as a key variable that affects the extraction process. A new 3D PTLC has recorded an improved resolution in the purification of flavonoid and the study has emphasised specifically the role of purified quercetin derivatives in an appreciable inhibition of amylase. The amylase inhibition by quercetin derivative has documented an effective control over physiological hyperglycaemia (after carbohydrate absorption) which can be considered as the first level of defence by an *in vivo* system to control T2DM and its post effects.

## Conclusion

The present study has highlighted the effectiveness of methanolic extract of *A. paeoniifolius* and purified

quercetin derivative in inhibiting amylase enzyme. The investigation also recorded a significant improvement in the flavonoid yield through CCD based RSM design. Finally, the overall result has highlighted the promising use of *A. paeoniifolius* as a good traditional food/ medicine to control T2DM. In future, studies can be extended to identify more flavonoid molecules through $MS^2$ and NMR based techniques. Similarly, functional food formulation based on *A. paeoniifolius* tubers can be developed which can have an economic and nutritive impact upon society.

## Conflict of interest

All authors declare no conflict of interest.

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