Original Article

Identification of Antioxidant and Antidiabetic Compounds in *Hedychium spicatum* using TLC Bioautography Coupled with Mass Spectrometry and their *in silico* Molecular Docking Studies

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ABSTRACT

Background: Traditional system of medicine has been practised since a long time to cure various diseases. More than 60-80 percent people across the world use herbal medicine for their health care. In the present study, TLC-MS bioautography combined with DPPH, α-amylase and α-glucosidase bioassay were used to compare antidiabetic and antioxidant activities in hydro alcoholic extracts of *H. spicatum* and also explore *in silico* molecular docking studies. **Materials and Methods:** In this paper, hydro-alcoholic extract of *H. spicatum* was prepared, run TLC and performed TLC- MS bioautography to identify antidiabetic and antioxidant bioactive compounds. **Results:** HPTLC analysis showed there are 10 and 5 metabolites present at 254 nm and 366 nm respectively in *H. spicatum*. Further, TLC-MS bioautography studies isolate total two lead compounds such as quercetin and beta-sitosterol. Quercetin shows DPPH, α-amylase and α-glucosidase activity whereas beta sitosterol shows α amylase activity only. **Conclusion:** In conclusion, TLC-MS bioautography proved to be a good technique to evaluate the antidiabetic and antioxidant potential of plant extracts and will be useful for discovering new therapeutic regimens.

Keywords: *Hedychium spicatum*, TLC-bioautography, Antioxidant, Antidiabetic, *in silico.*

INTRODUCTION

Since long times, medicinal plants are an enormous source of bioactive compounds with health-regulating activities. A traditional medicinal system like Ayurveda, Siddha, Unani and Chinese are still promising and has been practised over 1500 years. They have established a range of defence mechanisms to manage oxidative stress and other lifestyle disorders like diabetes. More than 60-80 percent people from different parts of the world depend upon traditional herbal medicine for their health promotion.[1] Several review and research articles have been published upon n-number of medicinal plants that possess specific pharmacological action on various ailments such anti-inflammatory, analgesic, antioxidant, antidiabetic, respiratory etc.[2]

Diabetes is a metabolic disorder, which is characterized by its most common condition, hyperglycaemia. It is usually grouped into two main types i.e. Type I diabetes mellitus, caused due to complete lack of insulin production and Type II diabetes mellitus due to the relative shortage of insulin secretion and tissue not responding to the insulin action. Modern diet pattern, obesity and sedentary lifestyle are some of the responsible factors for the disease to occur all over the world.[3] The risk of several complications of this disease leads to increased mortality rate. The most common life-threatening complications include nephropathy, retinopathy hypertension and other cardiovascular diseases. These health related issues lead to search novel bioactive

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compounds for new defensive measures as well as to decrease severe death rate associated with the disease.[4]

The oxidative stress is created by free radicals produced in the body; mainly reactive oxygen species (ROS) causes tissue damage from this. These free radicals are accountable for various diseases such as diabetes, inflammation, cancer, atherosclerosis and age-related health issues. Natural antioxidants found in medicinal plants have the ability to scavenge free radicals in the human body. These type of compounds play an important role in neutralizing the effect of free radicals.[5] Natural antioxidants have gained popularity across the globe as they are found to be harmless to the body. In addition to therapeutic properties such as anti-inflammatory, anticarcinogenic, antidiabetic and anti-Alzheimer activities, antioxidant activity can be a special function to obtain better health supplements with least side effects. In the present scenario, herbal medicines are gaining world-wide acceptance in the form of natural antioxidants with least side effects for use in food as well as pharmaceutical industry.

Hedychium spicatum Buch. Ham.exD.Don. generally pronounced as 'spiked ginger lily', 'Van haldi' or 'Kapoorkachari' is considered as one of the important species for its medicinal and food value.[6] The species belong to family Zingiberaceae. The species is native to south-eastern Asian countries in temperate and subtropical areas found within an altitude of 1000–2800 m asl. Traditionally the herb is widely employed in treating asthma, diarrhoea, inflammation, pain, bronchitis and blood diseases. It is being used in the traditional as well as in modern medicine, cosmetic and perfumery industries.[7] The overexploitation from wild for essential oil has put the species in the vulnerable and rare categories.

A number of analytical methods such as chromatography and spectroscopy have been developed for standardization of herbal products which include liquid chromatography, planar chromatography, gas chromatography, mass spectrometry high performance liquid chromatography (HPLC), and high-performance thin layer chromatography (HPTLC) to assure their quality, safety and efficacy.[8]

TLC- MS bioautographic hyphenated analysis have been usually done for qualitative analysis of antidiabetic and antioxidant compounds through coupling with DPPH, α-amylase and glucosidase bioassay. DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay is a common method to assess antioxidant capacity of natural compounds of plant origin. Oxidative stress is associated with the progression of disease complications. It has been found that antioxidant regimen combined with other therapeutic regimens may be a better approach to prevent the complications due to oxidative stress.[9] The *α*-amylase carries out breakdown of starch in the gut and glucose is released which finally reaches the blood circulation. Hence, amylase inhibitiors decrease the breakdown of starch in gut and ultimately reduces its absorption.[10] *α*-Glucosidase yields an aglycone and a glycone unit after hydrolysis of saccharides and heterosides. Therefore, inhibition of glucosidase enzyme decreases the release of glucose from saccharides and finally lowers the post-prandial levels of glucose in diabetic individuals.

Hedychium spicatum was judiciously chosen for the current study. Our aim is to evaluate the antioxidant and antidiabetic bioactive metabolites present in hydro alcoholic extracts of *H. spicatum* by using hyphenated TLC-MS bioautography techniques and also explore *in silico* molecular docking studies.

MATERIALS AND METHODS

Collection and identification of plants

Fresh rhizome of *Hedychium spicatum,* Himkachari was obtained from IHBT (CSIR), Palamapur (HP), and taxonomic identification was confirmed from National Medicinal Plant Board, New Delhi.

Reagents and Chemicals

Instrumentation details required for the study viz. HPTLC (CAMAG, Muttenz, Switzerland), HPTLC plates, Silica gel 60 F_{254} (Merck, Dermstadt, Germany), Water's ACQUITY UPLC(TM) system (Waters Corp., MA, USA) was utilized for mass spectrometry (MS) analysis, reverse phase column (C_{18} 1.7 µm, 2.1 x 100 mm; ACQUITY UPLC(R) BEH), Mass Lynx V4.1 (Waters, USA), α-amylase (CAS Number: 9000-90-2), HPLC grade water, α-glucosidase (CAS Number:9001-42-7), fast blue (CAS Number:14263-94-6) and pNPG (CAS Number:2207-68-3) were procured from Sigma Aldrich Co. (St Louis, USA).

Extract Preparation

Hedychium spicatum

The sample preparation was as follows: 40g of drug was transferred in 1000ml conical flask containing 400 ml of water and alcohol mixture (1: 1) and then transferred in Soxhlet apparatus and carried out for 6 hr. After completion of 6 hr Soxhlation, collected 3gm of residue, dried it and stored at a cool place for further uses.

Estimation of total phenolic content (TPC)

TPC was estimated by Folin-Ciocalteu Reagent (FCR) method in the hydroalcoholic extract of *H. spicatum.*[11] To determine the phenolic content, calibration curve of

gallic acid (standard) in different aliquots were prepared for establishing the correlation with test extract. The regression equation of calibration curve was utilized for the determination of TPC and expressed as milligrams of gallic acid equivalent per gram of dried extract.

Estimation of total flavonoid content (TFC)

TFC was determined by the colorimetric method by utilizing aluminium chloride. Standard calibration curve was prepared by using Quercetin as standard. The drug extract mixed with aqueous $AlCl_3$ (20% w/v) was used for preparing sample aliquot. After 10 min of incubation period absorptions were taken at 425 nm against a blank.[12] The flavonoid content of hydro alcoholic extracts of *H. spicatum* was estimated from the calibration curve of quercetin by using its different aliquots ranging from 10 to 100 µg/mL. The data were presented as mg of standard equivalent/ gm of samples.

HPTLC Analysis

The pre-coated aluminium TLC plates were made into 10×10 cm (Merck, Germany) by pressing against hard surface. Then prepared solution was centrifuged and supernatant was collected. Further supernatants were filtered through 0.22µm syringe filter and then CAMAG Linomat-V applicator was used for sample application on TLC plate, maintaining 10 mm from below, 15 mm distance from each side and a minimum 2 mm distance between each track at optimum temperature in specific developed solvent system for *H. spicatum* (toluene: ethyl acetate: acetic acid; 5.5: 4: 0.5 v/v/v) respectively. The plate was then transferred in the development chamber. After 40 min the developed plate was removed, air dried and scanned at 254 and 366 nm. Same TLC plate was used for TLC-bioautography MS analysis.

In vitro DPPH assay

The DPPH assay of the *H. spicatum* was carried out by the spectrophotometric method for the presence of DPPH as a free radical. The extract was mixed with DPPH solution and incubated in dark area at room temperature for ½ hr. Finally the absorbance was taken at 517 nm against ascorbic acid (positive control). Following equation has been used to calculate the percentage of inhibition.[13] Ascorbic acid used as standards for comparison.

$$PI = (1\text{-}A_{sample}/A_{control}) \times 100 \quad (1)$$

Where, A_{sample}, absorbance of test sampleand$A_{control}$, Absorbance of control.

Bioautography screening for antioxidant activity

The antioxidant potential of *H. spicatum* extract was established by using DPPH assay. After applying the sample on TLC plates with the help of CamagLinomat-V applicator, the plates were developed in twin glass HPTLC chamber. Then remove the TLC plates from the solvent chamber and air dried. After air dried TLC plates were sprayed with a solution of 5mM DPPH. Appearance of cream colour or bright yellowish bands against purple background, indicating that the bands area is active as an antioxidant.[9]

Bioautography screening for anti-diabetic activity

α-Amylase

A solution of alpha-amylase was prepared in buffer solution. Thereafter starch and iodine solution were prepared at room temperature for analysis. Iodine solution was kept at dark place. The samples of *H. spicatum* extract was applied on TLC silica gel F_{254} plates, plates were removed from development chamber and air dried. Further TLC developed plates were sprayed with enzyme solution and kept in desiccator for 1.5 hr. After this the plate was removed and treated with starch solution and kept for another 15 min for enzyme substrate reaction, then air dried and finally iodine solution was applied. The visibility of alpha-amylase activity on TLC plate was revealed as white-yellow spot against a dark background.[10]

α-Glucosidase

A solution of α-Glucosidase was prepared in buffer. *p*-NPG was dissolved in 50% aqueous ethanol and fast blue in HPLC grade water to prepare substrate solution. The samples of *H. spicatum* extract was applied in the band form on TLC silica G_{60} F_{254} plates using automatic sample applicator and developed in suitable mobile phase as used for finger printing analysis. After moving solvent upto 80mm, TLC plates were removed and air dried. Then the enzyme solutions were sprayed and shifted to desiccator for 2 hr. After 2 hr., the same TLC plates were removed and dipped in substrate solution (*in situ p*-NPG: fast blue in ratio 1:1). The visibility of α-Glucosidase inhibition on TLC plates was revealed as white spot against purple/violet background within 5-10 min.[13] A subsequent co-TLC was also carried out for each sample for isolation of respective bioautograms from active plates.

Analysis of bioactive metabolites by Mass Spectrometry

Areas corresponding to the DPPH, alpha-amylase and alpha-glucosidase inhibition zones on a bioautogram

were marked on the duplicate chromatogram and scraped the encircled zones with specially designed scrapper and dissolved in methanol. 0.22 µM syringe filter was used to filter prepared sample solution for MS analysis. MS was carried out on Water's ACQUITY UPLC(TM) system equipped with an auto-sampler, a column manager, a binary solvent delivery system and a tunable MS detector. Empower software has been used for data acquisition and it was executed in positive as well as negative manner. A mobile phase consisting methanol: water: formic acid (8:2:0.1%v/v) was used for achieving complete separation via reverse phase C_{18} column with flow rate 10 µL/min and split ratio 1:5 at optimum temperature. Gradient elution was followed for separation of samples. The flow rates (500 L/h) and (50 L/h) were fixed for nebulizer and cone gas respectively at a source temperature of 100°C. The cone voltage (40 KV) and capillary voltage (3.0 KV) were maintained. An inert gas (Argon) was selected for collision at a pressure of 5.3 x 10-5 Torr. For estimation of molecular mass and molecular composition of precursor ions and fragment ions Mass Lynx ver. 4.1 software was used and it was also used for metabolomics analysis of the samples.[14] The metabolites isolated from different samples were recognized by their m/z ratio obtained from MS data bank.

In silico analysis

Identification and selection of the appropriate drug target or receptor is the first step in the *in silico* drug designing procedure. Molecular docking was carried out with Autodock4.2 to study the nature of interaction, binding modes and selectivity of amylase and glucosidase receptors with quantified secondary metabolites of hydro alcoholic extract of *H. spicatum*. The alpha amylase crystal structure was procured from RCSB (Research Collaboratory for Structural Bioinformatics) protein data bank. The ligands namely quercetin and beta-sitosterol for *H. spicatum* were downloaded as 3D structure SDF file from PubChem (http://pubchem.ncbi.nlm.nih.gov/) and optimized using Ligands Input in the AD 4.2.

The target enzyme was prepared by adding up all polar hydrogen atoms with the AutoDock tools and merging its non-polar hydrogen atoms along with the removal of water molecule and other heteroatoms to the target enzyme, an essential step for partial atomic charges computation. Three-dimensional affinity grids (126 × 126 × 126Å) were considered for each atom.[15] The optimized ligand molecules were docked with refined diabetes receptors using autodock 4.2. The docking results were analyzed using PyMOL molecular graphics visualization tool. The docking of the ligands into the active site of alpha amylase and glucosidase were carried out using autodock 4.2. The best conformation with minimum binding energy was selected after completion of docking searches. PyMOL Molecular Graphics Visualizer 1.7.4.5 was used to analyze the interaction of docked protein-ligand complex conformations.

RESULTS

The main objective of the study was to identify bioactive metabolites present in hydro alcoholic extracts of *H. spicatum* having antioxidant and antidiabetic activities. Keeping in view, a developed method of TLC-bioautography MS was performed. The extracts were filtered and evaporated to dryness. Percentage yield was calculated as 21% for *H. spicatum*.

Total Phenolic and Flavonoid Content

In this study, the number of total polyphenols for *H. spicatum* was calculated from the calibration curve of the standard molecule as gallic acid (R^2= 0.9969) and quercetin (R^2=0.9921). The total phenolic and flavonoid content were found to be 34.12 and 21.35 mg of gallic acid and quercetin respectively in hydro alcoholic extracts of *H. spicatum*. The standard curve of gallic acid and quercetin were depicted in Figure 1.

HPTLC fingerprint analysis

Medicinal plants contain a variety of bioactive compounds having therapeutic activity against several ailments and are responsible for their pharmacological action. Hence, finger printing analysis of such plant extracts is very valuable and broadly used for its qualitative and quantitative estimation. Different R*f* values correspond to different chemical compounds. The HPTLC finger printing of hydro alcoholic extract of *H. spicatum* was produced on TLC plate and visualized at 254 and 366 nm. The analysis showed a maximum number of compounds viz. *10 and 5* at 254 and 366 nm *respectively* (Table 1 and Figure 2).

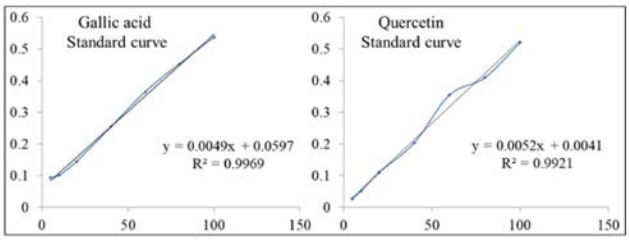

Figure 1: Standard calibration curve of gallic acid and quercetin.

Table 1: Data pertaining to HPTLC fingerprint of aqueous extract of *H. spicatum* at 254 and 366 nm.

S. No	Retention Time	Percentage Area	
		254	366
1	0.03	+	-
2	0.05	+	-
3	0.10	+	+
4	0.14	+	-
5	0.19	+	-
6	0.23	+	-
7	0.41	+	+
8	0.47	+	+
9	0.61	+	+
10	0.65	+	+
Total (10)		10	5

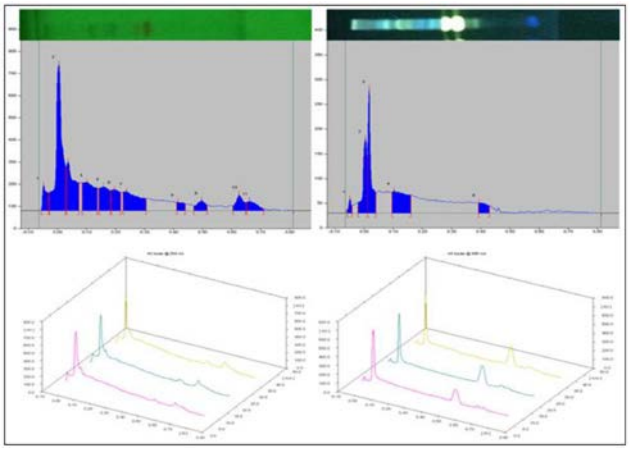

Figure 2: TLC photography and chromatogram of aqueous extract of *H. spicatum*at 254 and 366 nm.

DPPH radical scavenging assay

The DPPH free radical scavenging results are shown in Figure 3 as comparable with known antioxidant compound ascorbic acid. From the analysis of Figure 3, we can conclude that the scavenging effect of *H. spicatum* shows the excellent scavenging activity as compared to ascorbic acid.

TLC-Bioautography assay of DPPH, α-amylase and α-glucosidase

Antidiabetic and antioxidant compounds found in hydro alcoholic extract of *H. spicatum* was separated on TLC plate using above mentioned solvent system and estimated bioactive compounds with their inhibition zones.

To screen the antioxidant capacity of *H. spicatum* extract, a direct TLC-DPPH assay was established.[16] After development on TLC plates, the compounds

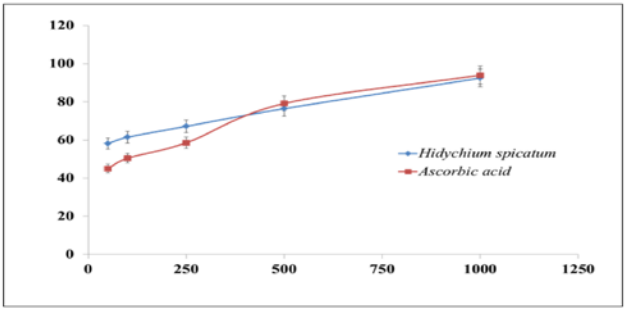

Figure 3: DPPH scavenging activity of *H. spicatum* with ascorbic acid.

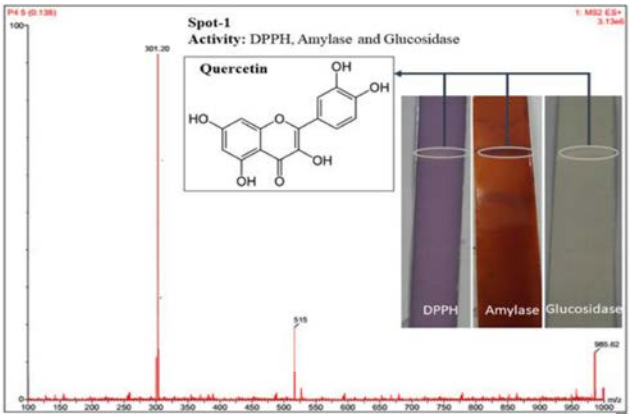

Figure 4: MS bioautogram of *H. spicatum*: spot 1: DPPH, α-amylase and α-glucosidase.

having radical scavenging action were estimated with DPPH reagent. After treatment with DPPH, bioactive bands were isolated from TLC plate and performed the mass spectrometry analysis. Mass spectrum revealed quercetin (m/z=302.23) responsible for antioxidant activity in case of *H. spicatum* (Figure 4).

Mass spectrometry of the scraped bioautogram of the TLC, revealed there are two bioactive compounds showed α-amylase inhibitory activity such as quercetin (m/z=302.23) and beta-sitosterol (Figure 4). The compounds such as quercetin (m/z=302.23) detected in *H. spicatum* by mass spectrometry also showed α-glucosidase inhibitory activity (Figure 5).

Docking interaction analysis

Computational screening of biomolecule libraries allows the identification of novel lead compounds accelerating the drug discovery process. *In silico* molecular docking was carried out to find out the increased activity of the bioactive compounds present in hydro alcoholic extract of *H. spicatum* on AD 4.2 using PyMol visualizer. The study showed binding interaction of ligands quercetin with both the receptors alpha-amylase and alpha-glucosidase while beta sitosterol showed

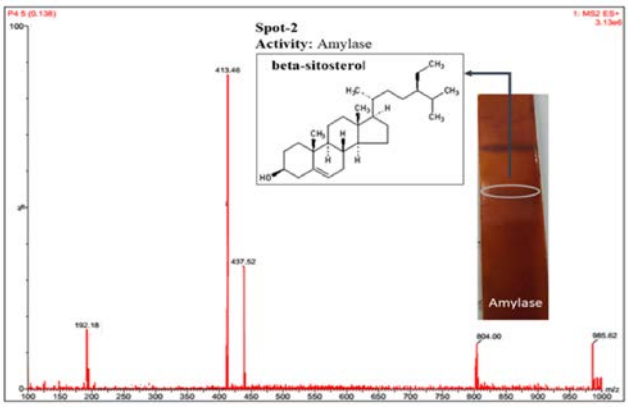

Figure 5: MS bioautogram of *H. spicatum*: spot 2: α-Amylase.

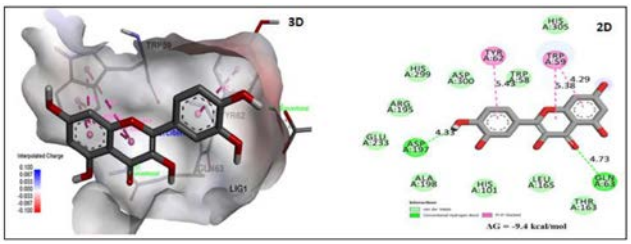

Figure 6: Docked pose of quercetin against α-amylase.

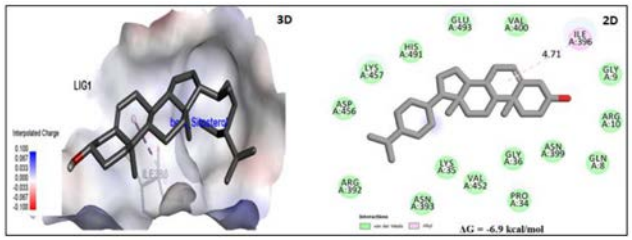

Figure 7: Docked pose of beta-sitosterol against α-glucosidase.

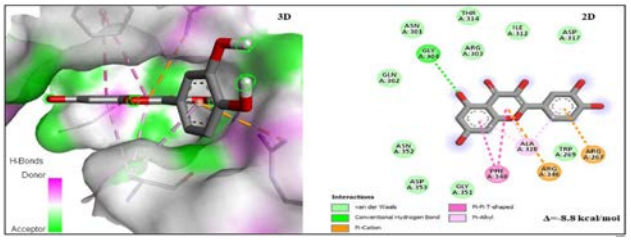

Figure 8: Docked pose of quercetin against α-glucosidase.

the interaction with alpha-amylase only. Docking studies revealed a number of amino acids of α amylase and glucosidase residue strongly bind to mentioned bioactive metabolites Figure 6, 7 and 8. Docking results of quercetin and beta-sitosterol against α-amylase, and α-glucosidase is shown in Table 2. Medicinal plants are promising source of phyto-pharmaceuticals used in traditional medicine to prevent various disorders. In this study, we determined and compared the docking scores of two compounds present in *H. spicatum* with different diabetic receptor using an automated docking model. α-amylase and α-glucosidase receptors represent the forefront of recent research and major advances have been made in understanding their molecular function of diabetes and its related disorders.

DISCUSSION

The extract contains pronounced amount of phenolics and flavonoids and are accountable for antioxidant and antidiabetic potential of the plant. Presence of free hydroxyl group present in flavonoids of extract are responsible for antioxidant activity. The free –OH group captures the free radicals and potentiate the antioxidant action. Phenolic and flavonoid (ArOH) compounds are well documented to decrease the rates of oxidation by donating a H atom to ROO* radicals.[17] By this

Table 2: Docking results of quercetin and beta-sitosterol against α-amylase, and α-glucosidase.

Sl. No	Compounds	Protien	Binding energy	H-bonds Interactions	Residual Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl Interactions
1	Quercetin	amylase	-6.4	Asp197(A), Glu63(A),	Glu223(A), Arg195(A), His299(A), Asp300(A), Tyr62(A), Trp58(A), Trp59(A), His300(A), Thr163(A), Leu165(A), His101(A), Ala198
2	Beta-sitosterol		-6.9	-	Asp456, Lys457(A), His491(A), Glu493(A), Val400(A), Ile396(A), Gly9(A), Arg10(A), Gln8(A), Asn399(A), Gly36(A), Pro34(A), Val452(A), Lys35(A), Asn393(A), Arg392(A)
2	Quercetin	Glucosidase	-8.8	Gly304(A)	Asn301(A), Thr314(A), Ile312(A), Asp317(A), Arg267(A), Trp269, Arg306, Ala310, Phe348(A), Gly351(A), Asp353(A), Asn352(A), Gln302(A)

mechanism, phenolics and flavonoids scavenge super oxides/ROS by stopping their spread as free radicals and play key role in reactive oxygen species (ROS) metabolism and prevent oxidation of biomolecules. A direct correlation between inflammatory responses, diabetes and free radicals generation, it is postulated that phenolic and flavonoids compounds protected the body against free radicals.[18]

The quality control studies play a key role to establish the pharmacological potential of the natural products. Therefore, a simple planar chromatography was performed to find out the number of secondary metabolites present in plant extracts. This is one of the best techniques to set the quality of natural products.

Outcome from this study revealed that *H. spicatum* extracts contain judicious amount of phenols and flavonoids which are having redox potential with antioxidant properties. The hydroalcoholic extract of *H. spicatum* had clearly shown the dose dependant antioxidant potential and maximum antioxidant potential was found at concentration1000µg/ml, which is almost equivalent to ascorbic acid. Besides scavenging the free radicals, phenolics and flavonoids curb the production of reactive oxygen species (ROS), quenched the trace elements and regulate antioxidant mechanism. Summarily, a strong therapeutic target is presumed to be generation of reactive oxygen species in the body. Natural compounds contain number of bioactive metabolites which inhibited free radical generation and enhance the defence mechanism of the body.[19] α-Amylase breaks the starch into saccharides and then releases the glucose and finally reaches the systemic circulation. The inhibition of amylase activity reduces the breakdown of starch in the GIT and glucose absorption subsequently. This will lead to a reduced glucose levels in the blood. The glucosidase is responsible for the hydrolysis of oligosaccharides and break the glycoside bonds to release the glucose.[20] Although, amylase and glucosidase inhibitors are of therapeutic interest in type 2 diabetes by inhibiting the breakdown and absorption of starch and carbohydrates from the gastrointestinal tract. Scoring function of molecular computational docking is a basic fundamental component. Successful examples showed that computational approaches have the one of the powerful approach to screen hits/leads from a huge database and design novel small molecules. Overall molecular docking results revealed quercetin is the best inhibition of α-glucosidasein molecular docking predictions as compared to α-amylase.[21] However, the realistic binding between bioactive molecules and receptors are still relied on experimental technology. In such cases, finger print profiling of the plant extracts is of great significance and commonly employed for its qualitative and quantitative evaluation. Different R*f* values characterize a number of chemical constituents.

CONCLUSION

The hydro alcoholic extract of *H. spicatum* had a remarkable anti-oxidant and anti-diabetic activities. TLC-MS bioautographic data revealed bioactive metabolites present in *H. spicatum* possess excellent antidiabetic and antioxidant activities. Overall, the studies illustrate that *H. spicatum* extracts have huge potential to be developed into an anti-diabetic drug. Thus, it could be further explored for its possible application in diabetes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ROS: reactive oxygen species; **GIT:** gastrointestinal tract; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl radical; **LC:** Liquid chromatography; **GC:** gas chromatography; **MS:** mass spectrometry; **TLC:** thin layer chromatography; **HPLC:** high-performance liquid chromatography; **HPTLC:** high-performance thin layer chromatography; **pNPG:** (4-Nitrophenyl-β-D- glucopyranoside).

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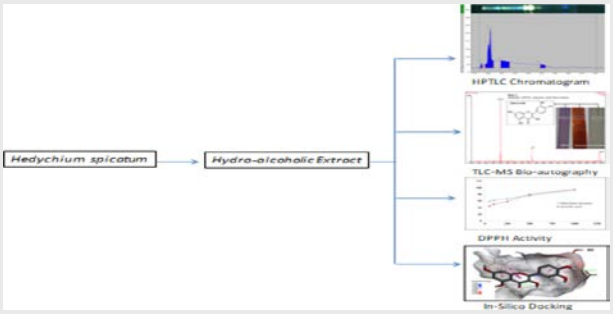
PICTORIAL ABSTRACT



SUMMARY

In this study, extraction and evaluation of hydro-alcoholic extracts of *Hedychium spicatum* for its antioxidant potential by DPPH method. HPTLC phytochemical screening revealed a number of metabolites present in the extracts. Further, by using TLC-MS bioautography, antioxidant and antidiabetic leads were also identified. Therefore, it can be summarized that *Hedychium spicatum* may prevent oxidative stress and diabetes.

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