



An investigation on in vitro anti-oxidant, anti-malarial and enzyme inhibitory activities of a few Indian *Piper* species and their metabolite profiling with in silico analysis

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Abstract

Detection of novel therapeutic agents from natural resources and to study their intervention in biomedical research are highly important. *Piper* species are of prime importance in commerce, economy and in a variety of ayurvedic applications. We investigated on a variety of bioactivities, viz. anti-alzheimer's, anti-malarial, anti-diabetic and antioxidant potentialities of 70% hydro-methanolic extracts of edible parts (leaf, stem and fruit) of four species of *Piper*, namely, *P. retrofractum*, *P. longum*, *P. nigrum*, indigenous to West Bengal and *P. betle* var. *Tympew* indigenous to Meghalaya, India. Leaves of *P. betle* var. *Tympew* showed very high activity both in Acetylcholinesterase inhibition, ($IC_{50} = 0.284 \text{ mg/mL} \pm 0.025$) and alpha glucosidase inhibition ($IC_{50} = 0.049 \text{ mg/mL} \pm 0.003$). In alpha amylase inhibition, the leaves of *P. longum* showed the highest inhibitory activity with an IC_{50} value of $2 \text{ mg/mL} \pm 0.257$. Fruits of *P. longum* showed highest activity in anti-malarial activity with IC_{50} value of $1.624 \text{ mg/mL} \pm 0.027$. The metabolomic variabilities of these plant parts and species using GC/MS assisted metabolomic tool were also determined and chemometrically we discriminated these species on the basis of their metabolome profiling. Quantification of piperine content of the plant parts were done using HPTLC. All the extracts showed significant anti-cholinesterase, anti-glycosidase, anti-malarial and antioxidant activities in a dose dependant manner. The bioactivities were compared with the standard drugs and in silico analysis established their efficacy as an alternative to synthetic drugs. Several metabolites detected via GC/MS showed strong anti-cholinesterase, anti-amylase, anti-glucosidase as well as anti-malarial activities.

Keywords *Piper* · Anti-oxidant · HPTLC · Piperine · In silico

Introduction

Piper is one of those genera which have diverse biological activities or potentialities—one of which is its' immense antioxidant potentialities (da Silva et al. 2017). Free radicals cause oxidative stress and most of the human diseases are caused due to oxidative stress resulting from free radicals, such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$) and non-radical species viz., hydrogen peroxide (H_2O_2), singlet

oxygen ($^1O_2$) and a variety of activated oxygen constituting Reactive Oxygen Species or ROS (Lobo et al. 2010). Oxidative stress is a phenomenon occurs due to imbalance between production and accumulation of ROS (Pizzino et al. 2017). A strong and active anti-oxidative defense system is required to balance the oxidative stress related disorders. The oxidative damage caused due to the production of free radicals is a critical etiological factor connected with several human metabolic disorders such as Type 2 diabetes, neurodegenerative diseases, cancers, rheumatoid arthritis, atherosclerosis, melanogenesis and several other age related disorders (Tonnis and Trushina 2017, Lobo et al. 2010).

Various parts of the species of *Piper* are edible and possess health improving properties. In traditional medicine, species of *Piper* are used to treat a variety of diseases such as urological problems, liver, skin and stomach diseases, for wound healing and as antipyretic and anti-inflammatory agents (Salehi et al. 2019). There are about 2000 species

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found in the genus *Piper*, making it the largest genera in the plant kingdom. The genus *Piper* is widespread in warm tropical and sub-tropical regions, with a significant number of species dominant in South-East Asia, Central and South America and some areas of Africa. The species are herbaceous plants or shrubs or small trees, typically aromatic because of the presence of essential oils (Rezod et al. 2024). Due to varied applications, *Piper* species has become very promising in diverse pharmaceutical studies. Pharmaceutically, *Piper* sp. have been used to treat inflammation, digestive disorders and respiratory diseases and the volatile oils and extracts from different *Piper* species are reported for their antimicrobial, antioxidant, anti-inflammatory and analgesic properties (Azman et al. 2023). Essential oil of *Piper rostratum* Roxb. demonstrated moderate antioxidant, acetylcholinesterase and lipoxygenase enzyme inhibition (Ramin et al. 2025). In India, *Piper longum* L., *Piper retrofractum* Vahl, *Piper betle* L. and *Piper nigrum* L. are well documented in traditional and folk medicine. These are the principal ingredients of many Ayurvedic formulations (Xiang et al. 2016). The fruits (except *P. betle*) are chiefly used for this purpose. But no significant systemic investigation has been done to examine medicinal properties as well as the phytochemical composition of leaves which are also edible and are used either as leafy vegetables or as masticatory (*P. betle*) in India, Bangladesh and Thailand.

Piper longum L., also known as “long-pepper” or “pip-pali” grows as an herbaceous vine or as a perennial shrub, widely distributed in Indian subcontinent, Sri Lanka, Middle—East and America. The fruits are mostly used as spice for culinary purposes, preservation and also as a strong remedy against cough and cold, bronchitis and other respiratory diseases (Biswas et al. 2022). *Piper nigrum* L. (Black pepper), commonly known as the “King of Spices”, used worldwide and is native to South India. Black pepper has been reported to have gastrointestinal property to increase appetite, also treats cough and cold, throat diseases, epilepsy, worms and piles (Kaliyaperumal et al. 2021). *Piper retrofractum* Vahl (Syn. *Piper Chaba* Hunter) also known as “Javanese long pepper” or commonly known as “Chui Jhaal” or “Choi Jhal” in the Bengal province of India and Bangladesh. It is a popular plant for spice and medicinal usage in Indonesia as well. The fruits are used for their anti-flatulent, expectorant, anti-tussive, appetizing and counter irritant properties in traditional medicine (Islam et al. 2020). *Piper betle* L. is a shade loving perennial vine. The different varieties of leaves are highly aromatic with different flavour and taste, are well-known as masticatory in South and South East Asian countries (Debnath et al. 2021).

Now-a-days enzyme inhibitors are used as major therapeutics for various diseases (Manivasagan et al. 2014). Here natural inhibitors of the enzymes like acetylcholinesterase and glycosidases (alpha-amylase and alpha-glucosidase) are

of special interest which are associated with neurodegenerative disorders and Type 2 diabetes respectively (Asano 2003; Houghton et al. 2006). It has been reported that the plant parts specially fruits of *P. longum*, *P. nigrum*, *P. retrofractum* and leaves of *P. betle* possess activity against these said enzymes (Valentao et al. 2010; Das and De. 2011; Kavitha et al. 2018; Nair et al. 2013; Perumal and Saravanabhavan 2018; Ingkaninan et al. 2003; Khatami et al. 2017; Xiang et al. 2017). As the plant parts are common ingredients of daily diet in India, Bangladesh, Indonesia and Thailand, these can serve as promising natural agents to treat these diseases (Chaveerach et al. 2006).

The enzyme acetylcholinesterase (EC 3.1.1.7) is one of the α/β serine hydrolases, plays significant role in the homeostasis of neurotransmission by continual termination of nerve impulses through the breakdown of acetylcholine to acetic acid and choline. The cholinergic pathway involves acetylcholine (ACh), acetylcholine transferase—the synthesizing enzyme, vesicular ACh transporter and the receptors—cumulatively these factors play the vital role of metabolic activities and cognitive abilities viz., visuo-spatial abilities, memory, learning, motor functions etc. (Yoon et al. 2022). The recent therapy for Alzheimer’s Disease (AD) and other neurodegenerative disorder is to inhibit AChE activity utilizing compounds which show structural similarity to ACh—the natural substrate, because cholinergic pathway is one route of interest to study the onset and development of the AD like neurodegenerative diseases. The drugs available such as rivastigmine, donepezil, tacrine, physostigmine are not very effective as symptoms are alleviated only in mild to moderate cases and these also exhibit several adverse side effects (Luo et al. 2021; Asen and Aluko 2022). So newer phytometabolites to inhibit AChE are required to be deciphered urgently.

Alpha-amylase (EC 3.2.1.1) and alpha-glucosidase (EC 3.2.1.20) are two enzymes which are members of the enzyme family called glycoside hydrolases. Alpha-amylase is involved in the hydrolysis of complex carbohydrates to oligosaccharides and disaccharides, which are again hydrolysed by alpha-glucosidase into glucose. Thus blood glucose level is increased which is the characteristic symptom of Diabetes Mellitus (Alagesan et al. 2012; Tundis et al. 2010). Acarbose, Miglitol, Voglibose are the enzyme inhibitors popularly used to control post-prandial hyperglycemia. Acarbose inhibits both the glycosidic hydrolases, but Miglitol and Voglibose inhibit α -glucosidase only. Long term usage of these inhibitors for the treatment of type 2 diabetes causes irritable gastrointestinal side effects (Poovitha and Parani 2016). So, identification of phytochemicals to inhibit the glycoside hydrolases could be of potential to treat or manage type 2 diabetes.

Malaria is a deadly tropical disease caused by the species of *Plasmodium* which are transmitted from one human



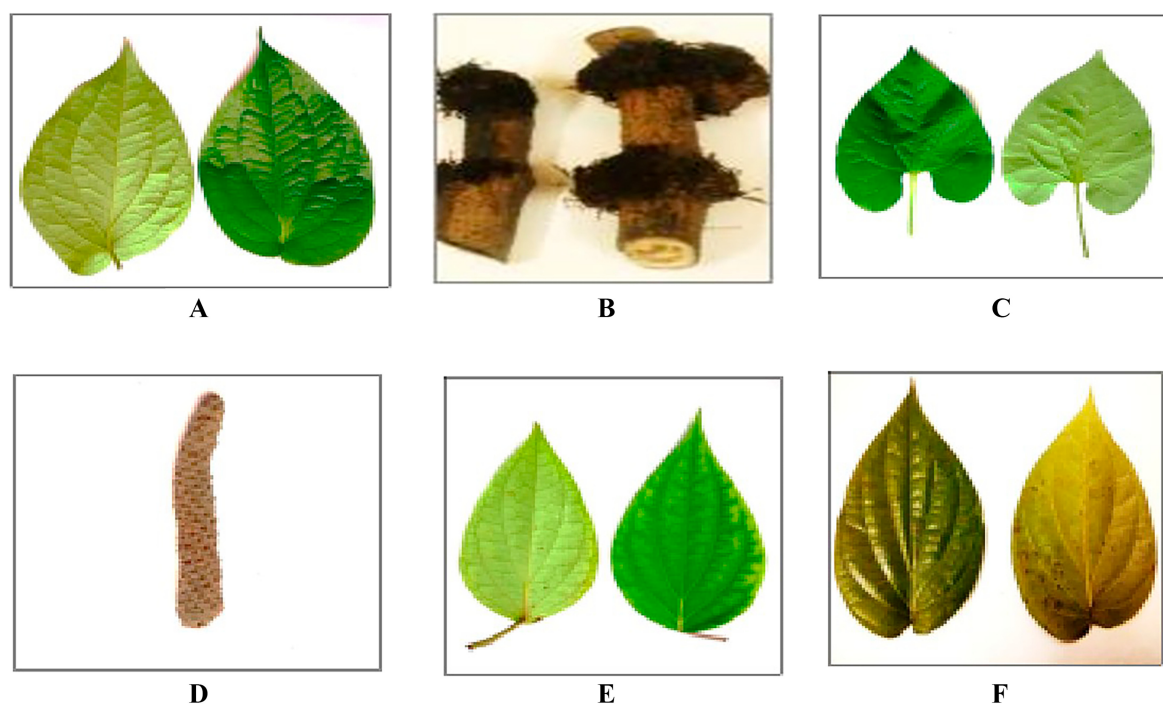


Fig. 1 A *P. retrofractum* leaf, B *P. retrofractum* stem, C *P. longum* leaf, D *P. longum* fruit, E *P. nigrum* leaf, F *P. betle* leaf

to another by infected female *Anopheles* sp. of mosquitoes. The rise of the conventional drug resistant parasite population is a great challenge to control this parasite. Therefore, plant resources must be explored in order to find new ways to eradicate the disease (Rifaie-Graham et al. 2018; Vanegas et al. 2018). Inside the RBCs, the protozoa, *Plasmodium*, digests haemoglobin in order to supply its own metabolism. The parasite releases heme: the non-digested molecule. Heme is highly toxic to the parasite as it disrupts the cell membrane of the parasite, resulting in cell lysis followed by self-digestion. To prevent this, the parasite neutralizes heme by biocrystallization, i.e., oxidation and dimerization of heme followed by self-assembly through H-bonding and Π (Pi) stacking into a triclinical crystal structure (Rifaie-Graham et al. 2018). Hemazoin crystallization in the inhibition of hematin self-assembly is the mechanism to detoxify heme. So, to discover new anti-malarial drugs is the need of the hour.

Plants produce a wide range of metabolites of diverse chemical groups. Therefore, it is necessary to explore the metabolites in order to interpret their underlying biochemical nature which can be related to their bioactivity. Furthermore, the non-targeted approaches of metabolite profiling have the potential to determine novel biomarkers. Interest in the edible plant species as natural sources of bioactive compounds has also augmented in recent years (Theodoridis et al. 2012; Saurina and Sentellas 2019; Ribbenstedt et al. 2018).

Materials and methods

Plant materials

Four species of the genus *Piper* (Family: Piperaceae), viz. *P. longum* L., *P. nigrum* L., *P. retrofractum* Vahl. were collected from West Bengal and *P. betle* L. var. *tymper* (a variety of Meghalaya) was collected from Meghalaya during August to September, 2018 (Fig. 1). Voucher specimens were prepared and submitted in the Calcutta University Herbarium (CUH) of Department of Botany, University of Calcutta (Table 1).

Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferrous chloride, ascorbic acid, FAME (Fatty Acid Methyl Ester) markers, methoxyamine hydrochloride, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (TMS), hematin (porcine), 1,2,4-benzenetriol, piperine, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and acetyl thiocholine iodide were purchased from Sigma-Aldrich. Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium carbonate, sodium hydroxide pellets, potassium dihydrogen phosphate, sulphuric acid, hydrochloric acid, glacial acetic acid, starch, 3,5-dinitrosalicylic acid, ortho-phosphoric acid, sodium chloride, potassium chloride, chloroform, methanol, sodium nitroprusside, dimethyl sulfoxide (DMSO), oleic acid, ribitol (adonitol)

Table 1 The four different *Piper* species collected for experiments

Sl. no.	Name	Vernacular name	Collection sites, districts, states	Accession no.	GPS location
1	<i>Piper retrofractum</i> Vahl.	Choi jhal	Bongaon, North 24 Parganas, West Bengal	20,107 [CUH]	Lat: 23° 02' 43.91" N Long: 88° 49' 51.02" E
2	<i>Piper longum</i> L.	Pipul	Garden of Medicinal Plants and Facilitation Centre, Narendrapur South 24 Parganas, West Bengal	20,106 [CUH]	Lat: 21° 56' 24" N Long: 88° 26' 24" E
3	<i>Piper nigrum</i> L.	Golmorich or black pepper	Garden of Medicinal Plants and Facilitation Centre, Narendrapur South 24 Parganas, West Bengal	20,096 [CUH]	Lat: 21° 56' 24" N Long: 88° 26' 24" E
4	<i>Piper betle</i> L.	Tympew paan	Shillong, Meghalaya	20,097 [CUH]	Lat: 25° 34' 8.11" N Long: 91° 52' 59.27" E

and glycine were purchased from Merck. Nitrobluetetrazolium (NBT), D,L-methionine, ammonium molybdate, acetylcholinesterase (source: electric eel), alpha-amylase (source: porcine), alpha-glucosidase (source: yeast), p-nitrophenyl-β-D-glucopyranoside (PNPG), ferrozine, sulphanilamide, naphthyl ethylene diaminedihydrochloride (NEDD), melezitose, benzoic acid, ferulic acid, quinic acid and gallic acid were purchased from Sisco Research Laboratory (SRL) Pvt. Ltd., Sodium acetate anhydrous, ethylene diamine tetra acetic acid (EDTA), riboflavin and 3,4-dihydroxy benzoic acid were purchased from Himedia. D-glucose was purchased from Qualigens Fine Chemicals.

Extraction procedure

The fresh clean leaves of *P. retrofractum*, *P. longum*, *P. nigrum* and *P. betle* were crushed using liquid nitrogen. The fresh raw fruits of *P. longum* were also crushed using liquid nitrogen. The raw stem of *P. retrofractum* was sun dried for a week. The dried stem powder of *P. retrofractum* and the crushed leaves and fruits of other *Piper* species were boiled in 70% methanol in a boiling water bath for 3 h with frequent stirring. Then the extracts were strained and evaporated to dryness using rotary evaporator under reduced pressure and temperature. Leaf extracts of *P. nigrum* and *P. betle*; leaf and fruit extracts of *P. longum*, and leaf and stem extracts of *P. retrofractum* were evaluated for their inhibitory capacity against the 3 enzymes, viz., acetylcholinesterase, alpha-amylase and alpha-glucosidase; and also to assess their antioxidant activities and anti-malarial potentialities. All the crude extracts were analyzed via GC–MS for untargeted metabolomic analysis.

In vitro assay for antioxidant activity

DPPH radical scavenging activity

The stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the extracts was measured in vitro following the method of Braca et al. (2001). Methanolic solutions of the crude extracts (0.1 mL) was mixed with 3 mL of 0.004% methanolic solution of DPPH. The reaction mixture was then incubated for 30 min at room temperature and then the absorbance was measured at 517 nm. The scavenging activity was measured based on the percentage of DPPH free radical scavenged and calculated as $(A_0 - A_e)/A_0 \times 100$ (A_0 = Absorbance without extract; A_e = Absorbance with extract).

Superoxide anion (O_2^-) scavenging activity

The assay to scavenge superoxide anion radical was carried out by the method described by Martinez et al. (2001) in the riboflavin-light-nitrobluetetrazolium (NBT) (Beauchamp & Fridovich 1971). Each reaction set (3 mL) contained phosphate buffer 50 mM of pH 7.8, methionine (13 mM), riboflavin (2 μM), EDTA (100 μM), NBT (75 μM) and methanolic solution of sample (1 mL). The reaction mixture was illuminated using a fluorescent lamp for 10 min. After 10 min of incubation, the absorbance of the product- the blue formazan was recorded at 560 nm. The percentage of superoxide radical scavenging ability of the extracts was determined using the equation $(A_0 - A_e)/A_0 \times 100$.

Total antioxidant capacity (TAC)

The TAC of the extracts was measured by phosphomolybdate method (Prieto et al. 1999). The reaction mixture contained sample solution (0.1 mL) and 1 mL of reagent solution (composed of phosphate buffer, sulphuric acid and ammonium molybdate in a ratio of 4:3:3) at acidic pH. The reaction was based on the reduction of Mo(IV) to Mo(V) by



the extract. The mixture was incubated for 90 min at 95 °C in a boiling water bath. The absorbance of the green phosphate/Mo(V) complex was measured at 695 nm against a blank (contained 1 mL of reagent solution and 0.1 mL of solvent, incubated under the same condition). Ascorbic acid was used as standard and the TAC was determined as Ascorbic acid equivalent (AAE) ($\equiv \mu\text{g Ascorbic acid}/\mu\text{g crude extract}$).

Metal chelating activity (ferrous ion)

The assay to chelate ferrous ion by the extracts was carried out following the method of Wang et al. (2003). Ferrozine can quantitatively form complexes with Fe^{2+} ion. In presence of plant extracts (chelating agent) the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Therefore, the measurement of colour reduction allows estimating metal chelating activity of the co-existing chelators. Fe^{2+} chelating ability is due to antioxidant capacity of the plant extracts. Reaction mixture containing 100 μL of extract, 200 μL of 0.5 mM ferrous chloride and 200 μL of 5 mM ferrozine were incubated at 37 °C for 10 min. After adding 1.5 mL of double distilled water to the reaction mixture, the absorbance was measured at 562 nm. The lower absorbance at 562 nm indicated stronger chelating effect. The chelating capacity was measured based on the percentage of Fe^{2+} chelated, calculated by the formula $(A_0 - A_e)/A_0 \times 100$.

Nitric oxide (NO) radical scavenging activity

Assay for Nitric oxide radical scavenging activity was performed modifying the method of Sumanout et al. (2004). At physiological pH, NO generated from aqueous sodium nitroprusside solution interacts with oxygen to produce nitrite ions, which was detected by Griess Ilosvay's reagent. Sodium nitroprusside (100 mM) (0.2 mL) dissolved in phosphate buffer saline (pH 7.4) (PBS) and different concentrations of the crude extract dissolved in PBS (1.8 mL) were incubated at 25 °C for 2.5 h. NO generated was detected by Griess reagent (2 mL). The pink chromophore formed during diazotization of nitrite ions with sulphanilamide and its subsequent coupling with naphthyl ethylene diaminedihydrochloride (NEDD) was measured at 546 nm. The percentage of NO scavenging ability of the extracts was determined using the equation $(A_0 - A_e)/A_0 \times 100$.

In vitro enzyme inhibitory activities

Acetylcholinesterase (AChE) inhibitory activity

The AChE inhibitory property was measured following the modified method of Ellman et al. (1961). The reaction

mixture contained 0.02 mL AChE (19.93 unit/mL buffer, pH 8), 0.01 mL aqueous extract, 1 mL buffer, 0.01 mL 0.5 mM DTNB, and 0.02 mL (0.6 mM) acetyl thiocholine iodide solution. The reaction mixture was incubated at 37 °C for 20 min. The yellow anion (5-thio-2 nitrobenzoate) produced in the reaction mixture was measured at 412 nm immediately. The percentage inhibition of AChE activity by plant extract was calculated as $(A_0 - A_e)/A_0 \times 100$, where A_0 = Absorbance without extract; A_e = Absorbance with extract.

Alpha-amylase inhibitory activity

Alpha-amylase inhibitory activity was assayed modifying the method of Bernfeld (1955). The reaction mixture consisting of different concentrations of the extracts (0.025 mL) and 0.05 mL of enzyme (ex. Porcine pancreas) (0.2 mL of 0.003% solution dissolved in 0.02 M phosphate buffer pH 6.9) was incubated at 37 °C for 20 min. Starch solution (0.025 mL) was added, and the mixture was again incubated at 37 °C for 3 min. Then DNSA (0.05 mL) was added to stop the reaction. The tubes were well plugged and heated at 100 °C in water bath for 5 min. After cooling, distilled water (1 mL) was added. The amount of reducing sugars released was measured at 540 nm spectrophotometrically. The percentage inhibition of enzyme activity was calculated as $(A_0 - A_e)/A_0 \times 100$.

Alpha-glucosidase inhibition assay

Alpha-glucosidase inhibitory activity was measured modifying the method of Kwon et al. (2008) using p-nitrophenyl- α -D-glucopyranoside as the substrate. Alpha-glucosidase (ex. microorganism) solution (0.006%) was prepared in 0.02 M phosphate buffer (pH 6.3). Each reaction mixture consisted of plant extract solution (0.034 mL), phosphate buffer (0.11 mL), and enzyme solution (0.034 mL). After 1 h incubation at 25 °C, p-nitrophenyl- α -D-glucopyranoside (0.17 mL) was added. The reaction mixture was again incubated at 30 °C for 30 min. The enzyme reaction was stopped by adding 1 M Na_2CO_3 solution (0.5 mL). Blank set consisted of 0.034 mL buffer instead of enzyme solution. The control set consisted of 0.034 mL of distilled water instead of plant extract solution. The optical density (OD) of the yellow colour produced due to p-nitrophenol formation was measured at 405 nm spectrophotometrically. The percentage inhibition of enzyme activity was calculated as $(A_0 - A_e)/A_0 \times 100$.

Haem-biocrystallisation and inhibition assay for potential anti-malarial activity

The potential anti-malarial activity of the plant extracts was evaluated modifying the protocol as reported by Hussain et al. (2013). 100 μL of plant extracts at a concentration of

0.3–12.5 mg/mL in 10% DMSO were incubated with 100 μ L 0.003 M hematin (dissolved in freshly prepared 0.1 M NaOH), 0.01 M oleic acid, 10 μ L of 1 M HCL. Then the reaction volume was adjusted to 1 mL using 0.5 M sodium acetate buffer at pH 5. Blank sets consisted of buffer without test compound i.e. plant extracts. The samples were incubated for 4 h with gradual shaking. After incubation, samples were centrifuged at 30,000 $\times$ g for 10 min at 21 $^{\circ}$ C. Then the supernatant was removed and the haemozoin pellets were re-suspended in 1 mL of 0.1 M NaOH and incubated for an hour at room temperature. Thereafter the samples were vortexed and the haemozoin content was determined by measuring the absorbance at 400 nm using a UV–VIS spectrophotometer (LMSP-UV1000B). The percentage inhibition of heme-biocrystallisation was calculated as $(A_0 - A_c)/A_0 \times 100$.

Gas chromatography–mass spectrometry analysis

Sample preparation for GC/MS analysis

5 mg of crude extract of the plant samples were taken in eppendorff tube and 1 mL of hydro-methanol solution (HPLC methanol and HPLC water mixed in a ratio of 1:1 v/v) was added. 20 μ L of 0.2 mg/mL ribitol (adonitol) solution was then added as an internal standard. Then the samples were cold centrifuged at 32,000 $\times$ g for 20 min. The aliquots were distributed in eppendorff tubes (150 μ L $\times$ 4) and evaporated to dryness. The residue obtained was re-suspended in 5 μ L of methoxyamine hydrochloride (20 mg/mL in pyridine) and subsequently agitated for 90 min at 30 $^{\circ}$ C. Then 45 μ L of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (TMS) was added and incubated at 37 $^{\circ}$ C with frequent agitation for 30 min to increase the volatility of metabolites. 1 μ L of FAME (Fatty Acid Methyl Ester) markers was added (prepared using FAMES of C₈–C₂₆ linear chain length dissolved in HPLC grade chloroform) before GC/MS analysis.

Temperature programme for GC/MS analysis

GC–MS analysis was performed using Agilent 7890A GC (software driver version 4.01 [054] equipped with 5795C inert MS with Triple Axis Detector. The analytical system consisted of HP-5MS capillary column (Agilent J & W; GC Columns [USA] measuring 30 m $\times$ 0.25 mm $\times$ 0.25 μ m). The method was described earlier by Kind et al. (2009), modified by Das et al. (2016) following users' guide. The analysis was performed with a run time of 37.5 min.

Oven temperature program: oven ramp 60 $^{\circ}$ C (1 min hold) to 325 $^{\circ}$ C at 10 $^{\circ}$ C/min, held for 10 min before cool-down. Other conditions such as injection temperature, the

MS transfer line and the ion source were maintained at 250, 290, and 230 $^{\circ}$ C respectively. The carrier gas used was helium (flow rate of 0.723 mL/min [Carrier linear velocity 31.141 cm/s]). Crude extracts were derivatized using methoxyamine hydrochloride and MSTFA. 1 μ L FAME marker (a mixture of Internal Retention Index [IRI] markers prepared using methyl esters of C₈ to C₂₆ linear chain length fatty acids dissolved in chloroform) was added to each of the samples. Derivatized samples (1 μ L) were injected via the split mode (Split ratio 10:1) onto the GC column. A solvent delay of 5.99 min was set to prevent overloading. Chromatographic peaks were identified after deconvolution using automated mass spectral deconvolution and identification system (AMDIS). Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra, retention times (RT), and retention indices (RI) with entries of mass spectra, RT and RI in Agilent Fiehn GC/MS Metabolomics RTL Library (2008) (Agilent Technologies, USA). Peak areas were normalized by dividing the value with extract dry weight in grams and the peak area of internal standard to obtain the relative response ratios (RRRs).

High performance thin layer chromatography (HPTLC) analysis

Chromatographic analysis was conducted on 20 $\times$ 10 cm² HPTLC plates of 0.25 mm thickness, pre-coated with silica gel 60 F₂₅₄. Crude extracts (at a concentration of 12 mg/mL HPLC grade methanol) along with the standard sample piperine (at a concentration of 0.5 mg/mL HPLC grade methanol) were applied as 6 mm wide bands by Linomat 5 applicator (Camag, Switzerland) equipped with a 100 μ L micro syringe. The applicator provided a delivery speed of 150 nl/s from the syringe with the help of nitrogen flow. The sample loaded plates were developed in previously saturated 20 $\times$ 10 cm² twin trough glass chamber (Camag) lined with filter paper at relative temperature (24 $\pm$ 2 $^{\circ}$ C), in the solvent system of toluene:ethyl acetate:diethyl ether (6:3:1, v/v) as mobile phase (Rajopadhye et al. 2011). After development for 15 min, the plates were dried at room temperature. Camag TLC scanner 4 was used to scan all the tracks. Absorption mode was set at λ = 337 nm. Spectra were recorded from 200 to 700 nm with the help of Deuterium (D₂) and Tungsten (W) lamp source. Slit dimensions were 6.00 $\times$ 0.45 mm, and scanning speed was 100 nm/s operated by winCATS software. Confirmation of the standard compound was done by superimposing the UV spectra of investigated samples and piperine standard. The amount of piperine present in crude extracts was quantified using the calibration curve of piperine (Table 2).



Molecular docking study

Gibbs free energy is important parameter that determines the ligand–protein interaction. Gibbs free energy is measured in terms of binding energy in AutoDockVina tools. AutoDockVina is an in-silico docking tool specialized in molecular docking studies. In the present study, a number of phytochemical constituents that have been screened in GC/MS analysing technique, have been further observed to demonstrate inhibition of enzyme activity in in-vitro experimental set up. Those phytochemical constituents that have been reported to demonstrate significant inhibitory potential in in-vitro experimental set up, are further observed in in silico docking tools in order to understand molecular binding interactions. Table 7 summarizes a list of various phytochemicals and their corresponding enzymes. Briefly, the phytochemicals (ligands) were downloaded from Pubchem as.sdf file, and were subsequently converted into.pdb file by an online tool, OpenBabel. Likewise the proteins were downloaded from Protein Data Bank as.pdb file. The proteins were further prepared for docking by removal of water, multiple domains and existing ligands in the protein crystals by Discovery Studio visualizer, whereas addition of polar hydrogen, Kollman and Gasteiger charges were done by AutoDockVina. All the ligands and proteins were converted into AutoDockVina compatible.pdbqt files.

Statistical analysis

At least three replicas were made in each experiment. Enzyme inhibitory activity is represented as mean % inhibition $\pm$ standard deviation (SD). Further regression equations were also prepared from this data, depending on which IC_{50} values were calculated. Multivariate analysis of the metabolites in leaves, stems and fruits of four *Piper* species was performed using the MetaboAnalyst 5.0—a comprehensive tool dedicated for metabolomics data analysis. Principal component analysis (PCA) was initially performed to detect intrinsic clustering. Partial least squares discriminant analysis (PLS-DA)-based regression analysis was then performed (Fig. 2).

Table 2 Quantification of piperine in following extracts based on HPTLC data

Sample extracts	Quantity (μ g/mL)
<i>Piper retrofractum</i> stem	3276.07
<i>Piper longum</i> fruit	3338.63

Results and discussion

Antioxidant activities

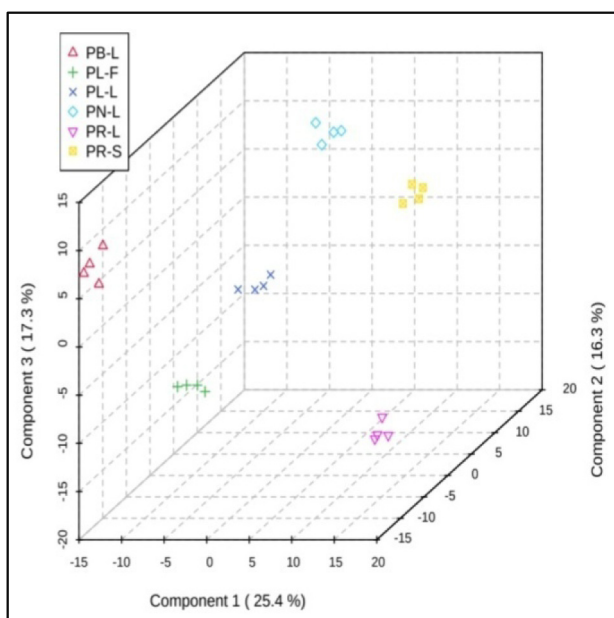
All the extracts scavenged DPPH, superoxide anion and nitric oxide free radical in a dose-dependent manner. Tympew variety of *Piper betle* leaf extract showed the highest ($IC_{50}=0.012 \pm 0.0002$ mg/mL) and *P. nigrum* leaf extract showed the lowest ($IC_{50}=0.246 \pm 0.015$ mg/mL) activity to scavenge DPPH. In case of superoxide anion radical scavenging activity, *P. betle* leaf extract again showed the highest ($IC_{50}=0.014 \pm 0.002$ mg/mL) and *P. retrofractum* stem extract showed the lowest ($IC_{50}=0.537 \pm 0.034$ mg/mL) activity. While in case of nitric oxide radical scavenging activity, *P. longum* fruit extract showed the highest ($IC_{50}=2.128 \pm 0.126$ mg/mL) and *P. longum* leaf extract showed the lowest ($IC_{50}=8.767 \pm 0.562$ mg/mL) scavenging activity. For metal (ferrous) ion chelating antioxidant property, *P. retrofractum* stem extract showed the highest ($IC_{50}=3.581 \pm 0.039$ mg/mL) and *P. longum* leaf extract showed the lowest ($IC_{50}=30.13 \pm 0.78$ mg/mL) chelation activity. All the plant extracts also indicated significant total antioxidant capacity (ascorbic acid equivalent) in a concentration-dependent trend. *P. betle* leaf extract showed the best total antioxidant capacity (0.61 ± 0.204 μ g ascorbic acid/ μ g crude extract) while other samples showed more or less similar total antioxidant capacity (Table 3).

Acetylcholinesterase inhibition

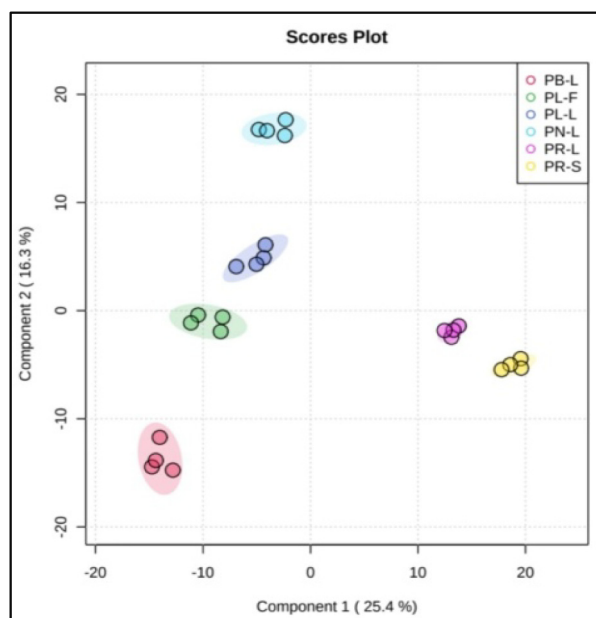
The hydro-methanolic extracts were tested for acetylcholinesterase inhibitory property. All the extracts inhibited acetylcholinesterase in a dose-dependent manner. *P. betle* leaf extract showed the highest ($IC_{50}=0.284 \pm 0.025$ mg/mL) inhibitory activity among the experimental samples and *P. longum* leaf extract showed the lowest ($IC_{50}=2.903 \pm 0.033$ mg/mL) inhibitory activity. Activities based on IC_{50} values of the extracts were compared with the established drug physostigmine which is used for inhibition of acetylcholinesterase and was earlier reported to have an IC_{50} value of 1.42 ± 0.02 μ M (Nag et al. 2015) (Table 4).

Alpha-amylase inhibition

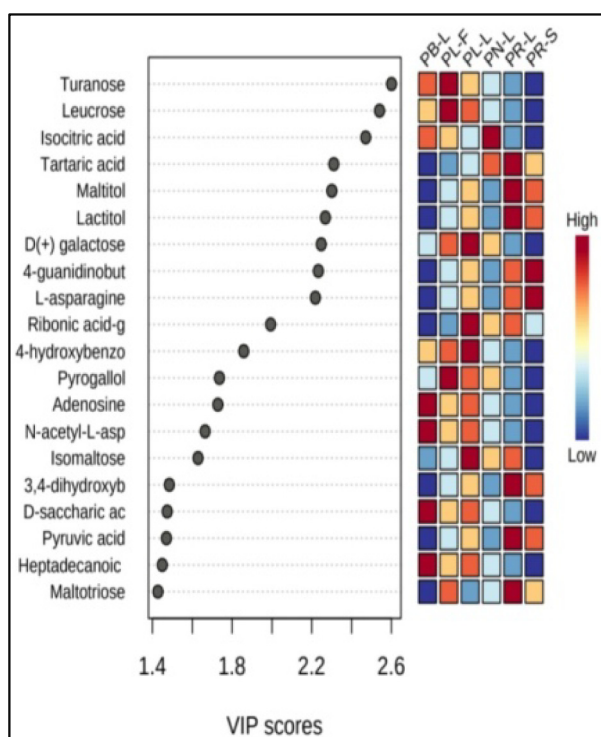
The aqueous-methanolic extracts were tested for alpha-amylase inhibitory activity. All the extracts inhibited the enzyme action in a concentration dependent manner. Among the extracts the highest inhibitory activity was showed by *P. longum* leaf ($IC_{50}=2 \pm 0.257$ mg/mL) and *P. retrofractum* stem showed the lowest inhibition ($IC_{50}=6.953 \pm 0.829$ mg/mL). Activities based on IC_{50} values of the extracts were



[a]



[b]



[c]

Fig. 2 **a** PLS-DA 3-D scores plot and **b** s-PLS-DA scores plot showing distinct groups of six different plant samples. **c** VIP scores in s-PLS-DA showing the top 20 features on the basis of metabolite

profile among the said samples. PB-L=*Piper betle* leaf, PL-F=*Piper longum* fruit, PL-L=*Piper longum* leaf, PN-L=*Piper nigrum* leaf, PR-L=*Piper retrofractum* leaf, PR-S=*Piper retrofractum* stem

Table 3 Antioxidant activities of the tested plant sample extracts

Extracts	IC ₅₀ (mg/mL) ± SD for DPPH radical scavenging activity	IC ₅₀ (mg/mL) ± SD for superoxide radical scavenging activity	Total Antioxidant Capacity (µg ascorbic acid/µg crude extract ± SD)	IC ₅₀ (mg/mL) ± SD for metal ion chelating capacity	IC ₅₀ (mg/mL) ± SD for nitric oxide radical scavenging activity
<i>Piper retrofractum</i> leaf	0.195 ± 0.003	0.229 ± 0.016	0.097 ± 0.018	9.38 ± 0.128	8.633 ± 0.267
<i>Piper retrofractum</i> stem	0.027 ± 0.002	0.537 ± 0.034	0.11 ± 0.021	3.581 ± 0.039	7.5 ± 0.087
<i>Piper longum</i> leaf	0.207 ± 0.008	0.18 ± 0.031	0.126 ± 0.017	30.13 ± 0.78	8.767 ± 0.562
<i>Piper longum</i> fruit	0.157 ± 0.011	0.427 ± 0.043	0.119 ± 0.016	18.653 ± 0.312	2.128 ± 0.126
<i>Piper nigrum</i> leaf	0.246 ± 0.015	0.275 ± 0.014	0.112 ± 0.047	29.514 ± 0.224	5.712 ± 0.474
<i>Piper betle</i> tympew variety leaf	0.0121 ± 0.0002	0.014 ± 0.002	0.61 ± 0.204	8.852 ± 0.117	6.815 ± 0.709

Table 4 Enzyme inhibitory activities of the tested plant samples, standard drugs and a few standard compounds negatively correlated with the enzyme inhibitions studied

Extracts	IC ₅₀ (mg/mL) ± SD for acetylcholinesterase inhibition	IC ₅₀ (mg/mL) ± SD for alpha-amylase inhibition	IC ₅₀ (mg/mL) ± SD for alpha-glucosidase inhibition
<i>Piper retrofractum</i> leaf	0.442 ± 0.028	6.15 ± 0.179	1.04 ± 0.051
<i>Piper retrofractum</i> stem	0.336 ± 0.022	6.953 ± 0.829	1.394 ± 0.109
<i>Piper longum</i> leaf	2.903 ± 0.033	2 ± 0.257	0.525 ± 0.013
<i>Piper longum</i> fruit	1.925 ± 0.129	2.524 ± 0.183	1.598 ± 0.023
<i>Piper nigrum</i> leaf	2.168 ± 0.018	3.373 ± 0.612	0.388 ± 0.022
<i>Piper betle</i> tympew variety leaf	0.284 ± 0.025	2.697 ± 0.429	0.049 ± 0.003
Standards			
Acarbose (standard drug)	NA	1.28 ± 0.00	0.009 ± 0.00
Physostigmine (standard drug)	1.42 ± 0.02	NA	NA
Piperine	0.837 ± 0.012	Could not be correlated	7.06 ± 0.094
1,2,4-Benzenetriol	28.55 ± 0.0007	2.06 ± 0.046	0.95 ± 0.002
3,4-Dihydroxy benzoic acid	8.56 ± 0.077	Could not be correlated	Could not be correlated
Benzoic acid	Could not be correlated	6.30 ± 0.184	Could not be correlated
Gallic acid	Could not be correlated	103.45 ± 2.54	Could not be correlated
Ferulic acid	Could not be correlated	Could not be correlated	2.37 ± 0.07
Quinic acid	Could not be correlated	Could not be correlated	10.77 ± 0.025
D-glucose	Could not be correlated	Could not be correlated	21.48 ± 0.313
D-(+) melezitose	Could not be correlated	Could not be correlated	128.60 ± 1.435
Glycine	Could not be correlated	Could not be correlated	312.64 ± 1.173

IC₅₀ values of extracts are expressed as mg/mL ± SD whereas that of the standard drugs and pure compounds are expressed as µM ± SD

compared with acarbose which is the standard drug used commercially to treat Diabetes mellitus type 2 and was earlier reported to have an IC₅₀ value of 1.28 ± 0.00 µM (Das and De 2012) (Table 4).

Alpha-glucosidase inhibition

The aqueous-methanolic extracts were tested for alpha-glucosidase inhibitory potentiality. All the plant extracts

showed enzyme inhibitory activities in a dose dependent mode. *P. betle* leaf extract showed the highest inhibition (IC₅₀ = 0.049 ± 0.003 mg/mL) against this enzyme while *P. longum* fruit extract showed the lowest inhibitory activity (IC₅₀ = 1.598 ± 0.023 mg/mL). Activities based on IC₅₀ values of the extracts were compared with acarbose which is the standard drug used commercially to treat Diabetes Mellitus type 2 and was earlier reported to have an IC₅₀ value of 0.009 ± 0.00 µM (Das and De 2012) (Table 4).

Anti-malarial activity

The aqueous-methanolic extracts were evaluated for anti-malarial assay. All the extracts showed dose dependent inhibition. Among all the extracts, *P. longum* fruit showed the best anti-malarial activity with an IC_{50} of 1.624 ± 0.027 mg/mL while *P. retrofractum* leaf showed the least activity ($IC_{50} = 5.648 \pm 0.034$ mg/mL) against the haem-biocrystallisation assay. During intra-erythrocytic development, malaria parasites degrade large amount of haemoglobin within a specified organelle—the digestive vacuole to supply amino acids for its' growth and development. A toxic by-product-ferriprotoporphyrin is produced during the catabolism of haemoglobin. Parasite detoxifies this by-product through the formation of insoluble haemozoin crystals. Activities based on IC_{50} values of the extracts were compared with the standard drug, chloroquine diphosphate which is used commercially to treat malaria and was reported earlier to have the IC_{50} value 163.78 ± 0.00 μ M (Chatterjee et al. 2023) (Table 5).

Analysis of phytochemical composition

GC/MS based metabolite profiling of 70% methanolic extracts detected a total of 128 metabolites including 16 amino acids, 25 organic acids, 12 fatty acids, 1 inorganic acid, 20 phenolic compounds, 1 alkaloid, 2 flavone and flavonoid glycosides, 40 sugar and their derivatives and 11 other compounds using Agilent Fiehn Metabolomics

Table 5 Antimalarial activity of the tested plant extracts, standard drug and a few standard compounds

Extracts	IC_{50} (mg/mL) $\pm$ SD
<i>Piper retrofractum</i> leaf	5.648 ± 0.034
<i>Piper retrofractum</i> stem	1.923 ± 0.049
<i>Piper longum</i> leaf	4.413 ± 0.011
<i>Piper longum</i> fruit	1.624 ± 0.027
<i>Piper nigrum</i> leaf	4.937 ± 0.014
<i>Piper betle</i> tympew variety leaf	3.244 ± 0.009
Chloroquine diphosphate (standard drug)	163.78 ± 0.00
1,2,4-Benzenetriol	2.22 ± 5.34
3,4-Dihydroxy benzoic acid	Could not be correlated
Benzoic acid	Could not be correlated
Gallic acid	1.00 ± 3.53
Ferulic acid	Could not be correlated
Quinic acid	Could not be correlated
D-glucose	Could not be correlated
D-(+) melezitose	Could not be correlated
Glycine	Could not be correlated

IC_{50} value of the standard drug Chloroquine diphosphate is expressed as μ M $\pm$ SD

Library. The semi-quantitative measurement of the identified compounds of all the studied samples is presented as response ratios $\pm$ SD (Table 6).

Piperine is one of the marker components of *Piper* species. The piperine was detected and semi-quantified via GC/MS. The quantification of piperine was done using HPTLC and the quantity measured was 3276.07 μ g/mL and 3338.63 μ g/mL in *P. retrofractum* stem and *P. longum* fruit respectively (Table 2). In all the other studied plant samples, piperine was not found in measurable quantity.

PLS-DA 3-D scores plot, s-PLS-DA scores plot based on the GC/MS based metabolomic study discriminated the study samples distinctly. Out of 128 metabolites, top 20 metabolites were screened on the basis of VIP scores in s-PLS-DA (Fig. 2a–c).

The differences in concentration of the top 20 metabolites determined from the overall co-efficient scores of PLS-DA are depicted in Fig. 3. It was observed that in fruits sample of *Piper longum*, sugars such as turanose, leucrose, D(+)-galactose, organic acid like isocitric acid, phenolic compounds such as pyrogallol and 4-hydroxy benzoic acid was found in highest concentrations. The leaf extract of *Piper longum*, also showed the abundant presence of isocitric acid, leucrose, D(+)-galactose, ribonic acid gamma lactone, pyrogallol, 4-OH-benzoic acid, isomaltose and 3,4-dihydroxy benzoic acid. Turanose is one of the structural isomers of sucrose and it is considered as a novel sweetener due to its mild taste and low calorie. So for type 2 diabetic patients this compound can be applied as functional sweetener or as sucrose substitute and also as bulking agent in food processing industries (Han et al. 2021). On the other hand, D-galactose is a reducing sugar, abundantly present in milk, fruits and vegetables. But it was reported (Budni et al. 2016) that oral administration of D-galactose in 4 month old rats caused cognitive impairment together with oxidative damage. Isocitric acid is an organic acid, an isomer of citric acid recommended as a potent antioxidant and promising compound in pharmaceutical and food industry. Isocitric acid could influence the spatial memory of intact laboratory rats (Morgunov et al. 2020). Pyrogallol is an organic compound derived from gallic acid and characterised by the presence of three hydroxyl (–OH) groups and classified within the phenol family. In a simulation of protein–ligand binding, pyrogallol was observed to interact with the active site of α -glucosidase. Also, the inhibitory effect of pyrogallol on α -glucosidase exhibited a mixed type of inhibition characterised by reversible and rapid-binding manner (Zheng et al. 2018). 4-hydroxy benzoic acid is reported as a hypoglycaemic metabolite mediated by an increase in the peripheral glucose consumption. 3,4-dihydroxy benzoic acid, also known as protocatechuic acid demonstrates both antioxidant and metal chelating capabilities. Moreover, derivatives of this compound have been assessed to



Table 6 Response ratios $\pm$ SD of the metabolites detected among the studied *Piper* species using GC/MS analysis

	Names of metabolites	Response ratios of compounds with standard deviation					
		<i>P. retrofractum</i> leaf	<i>P. retrofractum</i> stem	<i>P. longum</i> leaf	<i>P. longum</i> fruit	<i>P. nigrum</i> leaf	<i>P. betle</i> leaf
Amino acids	Aspartic acid	15.81 $\pm$ 12.51	15.94 $\pm$ 5.70	14.50 $\pm$ 10.40	–	5.35 $\pm$ 5.80	0.65 $\pm$ 0.37
	Beta-alanine	3.31 $\pm$ 2.87	0.94 $\pm$ 0.68	15.08 $\pm$ 23.94	2.00 $\pm$ 1.57	2.80 $\pm$ 3.18	0.79 $\pm$ 0.58
	Beta-cyano-L-alanine	–	0.18 $\pm$ 0.08	–	–	–	–
	DL-isoleucine	0.53 $\pm$ 0.50	–	–	–	–	–
	Glycine	–	–	0.40 $\pm$ 0.47	–	–	–
	L-alanine	–	5.26 $\pm$ 3.06	0.70 $\pm$ 0.94	–	1.64 $\pm$ 0.99	–
	L-asparagine	9.04 $\pm$ 7.91	17.19 $\pm$ 11.43	–	–	–	–
	L-glutamic acid	36.02 $\pm$ 41.01	15.33 $\pm$ 30.24	25.49 $\pm$ 34.38	17.75 $\pm$ 7.51	21.69 $\pm$ 15.17	20.63 $\pm$ 14.11
	L-mimosine	–	0.04 $\pm$ 0.03	–	1.78 $\pm$ 0.78	–	–
	L-proline	–	0.92 $\pm$ 1.22	–	0.92 $\pm$ 0.34	–	–
	L-pyrogutamic acid	61.39 $\pm$ 31.14	36.60 $\pm$ 11.30	22.07 $\pm$ 29.16	–	30.01 $\pm$ 26.11	39.47 $\pm$ 30.11
	L-serine	4.91 $\pm$ 3.12	1.75 $\pm$ 0.23	2.89 $\pm$ 3.92	–	2.28 $\pm$ 2.00	0.43 $\pm$ 0.19
	L-threonine	0.59 $\pm$ 0.97	0.17 $\pm$ 0.20	–	–	–	–
	L-valine	1.34 $\pm$ 1.20	0.46 $\pm$ 0.55	–	0.80 $\pm$ 0.95	–	–
	N-acetyl-L-aspartic acid	–	–	–	–	–	6.46 $\pm$ 2.43
Organic acids	Tyramine	–	0.50 $\pm$ 0.23	–	–	–	–
	Citraconic acid	–	0.59 $\pm$ 0.13	–	6.07 $\pm$ 8.95	–	0.73 $\pm$ 0.34
	Citramalic acid	–	–	–	0.96 $\pm$ 0.55	–	–
	Citric acid	413.97 $\pm$ 196.22	232.73 $\pm$ 66.11	1167.30 $\pm$ 953.85	3383.35 $\pm$ 1559.36	1407.57 $\pm$ 722.97	303.91 $\pm$ 247.78
	4,8-Dihydroxyquinoline-2-carboxylic acid (xanthurenic acid)	–	0.25 $\pm$ 0.17	–	–	–	–
	D-malic acid	339.46 $\pm$ 142.18	204.88 $\pm$ 146.97	1861.29 $\pm$ 1835.48	2232.03 $\pm$ 602.87	666.39 $\pm$ 284.98	82.73 $\pm$ 79.79
	Fumaric acid	26.57 $\pm$ 16.50	17.75 $\pm$ 3.53	55.04 $\pm$ 31.23	56.89 $\pm$ 14.70	7.72 $\pm$ 6.38	8.00 $\pm$ 3.38
	Glyceric acid	65.24 $\pm$ 42.25	15.48 $\pm$ 3.63	4897.56 $\pm$ 3139.67	925.25 $\pm$ 345.75	49.88 $\pm$ 28.78	88.14 $\pm$ 52.58
	Glycolic acid	55.26 $\pm$ 29.57	37.33 $\pm$ 9.07	161.93 $\pm$ 105.99	354.60 $\pm$ 121.17	40.00 $\pm$ 26.79	44.22 $\pm$ 16.97
	3-Hydroxy-3-methylglutaric acid(dicrotic acid)	–	1.10 $\pm$ 0.71	42.06 $\pm$ 39.75	6.10 $\pm$ 2.59	–	–
	Isocitric acid	–	–	–	1347.94 $\pm$ 1047.80	507.16 $\pm$ 593.05	223.52 $\pm$ 181.68
	2-Isopropylmalic acid	–	232.73 $\pm$ 66.11	–	–	–	$\pm$
	Itaconic acid	–	0.25 $\pm$ 0.17	7.09 $\pm$ 5.19	14.02 $\pm$ 4.03	2.34 $\pm$ 2.19	4.37 $\pm$ 2.87
	L-(+) lactic acid	97.10 $\pm$ 57.92	204.88 $\pm$ 146.97	117.88 $\pm$ 67.00	1357.93 $\pm$ 489.62	41.75 $\pm$ 12.83	87.61 $\pm$ 30.32
	Maleamic acid	2.44 $\pm$ 1.22	17.75 $\pm$ 3.53	–	–	–	0.65 $\pm$ 0.18
	Maleic acid	2.89 $\pm$ 1.57	15.48 $\pm$ 3.63	5.78 $\pm$ 8.26	116.73 $\pm$ 34.76	4.62 $\pm$ 5.52	13.63 $\pm$ 14.29
	Malonic acid	42.98 $\pm$ 25.38	37.33 $\pm$ 9.07	119.69 $\pm$ 63.17	23.08 $\pm$ 8.19	22.63 $\pm$ 29.97	2.95 $\pm$ 0.97
	Oxalic acid	20.68 $\pm$ 12.57	1.10 $\pm$ 0.71	59.72 $\pm$ 39.34	172.94 $\pm$ 192.29	3.62 $\pm$ 2.02	1.74 $\pm$ 1.17
	Picolonic acid	0.14 $\pm$ 0.17	–	–	–	–	–
	Pipecolic acid	–	6.98 $\pm$ 12.63	–	–	–	–
	Pyruvic acid	0.72 $\pm$ 0.38	0.66 $\pm$ 0.48	–	–	–	–
	Succinic acid	77.00 $\pm$ 43.05	33.78 $\pm$ 12.30	127.84 $\pm$ 94.01	240.80 $\pm$ 82.70	65.80 $\pm$ 46.17	22.56 $\pm$ 9.26
	Tartaric acid	16.34 $\pm$ 6.78	1.92 $\pm$ 1.49	–	–	8.77 $\pm$ 8.38	–
	Tartronic acid	1.51 $\pm$ 1.25	1.80 $\pm$ 2.61	4.22 $\pm$ 4.38	–	–	–
	Trans-aconitic acid	–	3.61 $\pm$ 1.98	–	21.67 $\pm$ 9.23	25.83 $\pm$ 10.71	36.06 $\pm$ 14.31
	Trans,trans-muconic acid	–	2.44 $\pm$ 1.11	–	45.18 $\pm$ 33.22	–	12.81 $\pm$ 6.88
Inorg. acid	Phosphoric acid	254.27 $\pm$ 108.25	77.02 $\pm$ 25.27	899.89 $\pm$ 630.12	676.43 $\pm$ 240.12	184.19 $\pm$ 247.26	68.75 $\pm$ 21.63

Table 6 (continued)

Names of metabo- lites		Response ratios of compounds with standard deviation					
		<i>P. retrofractum</i> leaf	<i>P. retrofractum</i> stem	<i>P. longum</i> leaf	<i>P. longum</i> fruit	<i>P. nigrum</i> leaf	<i>P. betle</i> leaf
Fatty acids	Capric acid	–	–	–	1.10 ± 0.87	–	–
	2-Furoic acid	4.90 ± 2.99	1.73 ± 0.47	1.47 ± 1.08	11.02 ± 8.94	2.58 ± 2.00	3.57 ± 1.38
	Glycerol	62.43 ± 106.99	11.80 ± 5.26	114.62 ± 114.53	746.80 ± 846.66	269.56 ± 411.82	5.01 ± 1.85
	4-Guanidinobutyric acid	10.67 ± 7.60	13.67 ± 6.85	–	–	–	–
	Heptadecanoic acid	–	–	–	–	–	0.82 ± 0.34
	2-Hydroxybutyric acid	–	0.22 ± 0.36	–	–	–	0.25 ± 0.10
	Lauric acid	0.37 ± 0.59	–	2.09 ± 3.16	4.82 ± 4.92	1.76 ± 0.32	–
	Linoleic acid	–	12.63 ± 2.03	–	7.85 ± 8.42	–	–
	Myristic acid	–	–	1042.12 ± 834.24	613.36 ± 285.77	308.26 ± 267.81	–
	Oleic acid	–	–	–	4.59 ± 8.57	–	–
Phenolic com- pounds	Palmitic acid	15.71 ± 12.08	17.58 ± 1.27	140.55 ± 93.83	234.45 ± 60.81	37.81 ± 28.89	29.37 ± 14.04
	Stearic acid	15.80 ± 9.39	13.53 ± 1.27	84.98 ± 54.26	244.61 ± 37.29	27.40 ± 18.60	36.89 ± 19.74
	Benzene-1,2,4-triol	–	–	–	–	–	0.31 ± 0.19
	Benzoic acid	0.85 ± 0.86	1.04 ± 0.19	1.71 ± 2.40	0.69 ± 0.25	0.28 ± 0.22	0.55 ± 0.25
	Caffeic acid	3.25 ± 1.70	–	7.68 ± 5.83	–	0.67 ± 0.63	1.24 ± 0.48
	Chlorogenic acid	218.77 ± 116.59	–	–	–	–	–
	Cinnamic acid	–	1.55 ± 0.90	–	–	–	–
	3,4-Dihydroxybenzoic acid	2.65 ± 1.24	1.29 ± 0.97	1.90 ± 2.87	–	–	–
	Ferulic acid	1.14 ± 0.65	1.45 ± 0.71	8.48 ± 7.03	–	0.25 ± 0.29	0.43 ± 0.12
	Gallic acid	–	–	32.90 ± 34.74	24.74 ± 8.88	6.53 ± 3.87	–
	Homogentisic acid	3.67 ± 7.22	–	–	–	–	–
	Hydrocinnamic acid	–	–	91.21 ± 86.91	–	–	–
	Hydroquinone	2.74 ± 4.18	0.09 ± 0.04	0.43 ± 0.55	–	0.15 ± 0.14	0.23 ± 0.10
	4-Hydroxybenzoic acid	–	–	2.80 ± 1.69	3.33 ± 2.38	1.17 ± 1.11	0.20 ± 0.07
	4-Hydroxycinnamic acid	9.62 ± 4.87	1.29 ± 0.76	14.16 ± 9.89	–	0.48 ± 0.35	4.54 ± 1.81
	4-Hydroxy-3-methoxybenzoic acid	0.35 ± 0.51	3.67 ± 0.48	–	2.04 ± 1.25	–	–
	4-Isopropylbenzoic acid	–	–	–	2.10 ± 0.95	–	–
	Pyrogallol	–	–	2.61 ± 1.91	2.78 ± 1.11	0.98 ± 0.80	0.44 ± 0.53
	Quinic acid	94.51 ± 65.04	–	–	–	291.20 ± 135.67	57.69 ± 46.10
	Shikimic acid	23.66 ± 40.57	–	–	–	24.80 ± 27.32	–
Sugars and sugar deriva- tives	4-Vinylphenol	–	1.52 ± 1.69	–	–	–	–
	Kaempferol	–	3.00 ± 1.86	–	–	–	–
	Vitexin	0.23 ± 0.14	–	–	–	–	–
	Acetol	0.72 ± 0.73	–	–	–	–	–
	Cellobiose	38.04 ± 43.62	–	–	37.02 ± 9.42	428.74 ± 489.28	–
	D-allose	412.82 ± 239.98	36.63 ± 38.30	1282.22 ± 1356.92	1926.68 ± 670.41	576.39 ± 854.34	22.26 ± 16.40
	D(+) altrose	–	25.26 ± 35.48	–	–	178.37 ± 162.77	86.65 ± 74.45
	D(+) galactose	–	–	411.10 ± 313.53	418.69 ± 441.75	142.16 ± 165.14	26.97 ± 24.43
	D-glucose	459.47 ± 219.21	86.38 ± 34.50	1401.08 ± 1307.75	522.75 ± 491.32	713.17 ± 674.58	144.58 ± 71.96
	D-mannitol	51.57 ± 21.21	17.41 ± 11.32	106.37 ± 93.45	1107.25 ± 561.60	11.41 ± 5.28	3.34 ± 1.78
	D-mannose	68.76 ± 62.22	14.12 ± 4.69	1150.54 ± 779.43	2661.95 ± 1724.40	700.95 ± 1068.36	28.18 ± 24.97
	D-(+) melezitose	45.70 ± 35.77	40.40 ± 7.00	362.68 ± 379.67	–	–	21.10 ± 24.02
	D-saccharic acid	–	–	–	–	–	1.04 ± 0.37
	D-threitol	23.91 ± 21.35	–	–	80.52 ± 19.27	25.29 ± 15.28	–
	D-(+) trehalose	3.82 ± 5.06	59.02 ± 62.97	14.87 ± 10.88	59.18 ± 32.65	–	12.10 ± 6.69
	Fructose	181.25 ± 114.10	81.48 ± 11.22	1252.99 ± 837.87	920.25 ± 687.00	455.82 ± 613.14	99.19 ± 50.56



Table 6 (continued)

	Names of metabolites	Response ratios of compounds with standard deviation					
		<i>P. retrofractum</i> leaf	<i>P. retrofractum</i> stem	<i>P. longum</i> leaf	<i>P. longum</i> fruit	<i>P. nigrum</i> leaf	<i>P. betle</i> leaf
	Galactinol	233.47 ± 198.56	20.36 ± 23.25	603.90 ± 421.99	49.19 ± 49.93	1138.91 ± 848.56	185.61 ± 61.61
	Gluconic acid	100.45 ± 51.10	175.04 ± 110.21	1228.64 ± 803.93	456.47 ± 59.55	49.04 ± 36.22	126.52 ± 168.16
	Gluconic acid lactone	–	12.30 ± 5.97	1293.81 ± 1451.88	–	–	10.62 ± 12.73
	Glycerol 1-phosphate	1.74 ± 0.98	2.47 ± 1.19	–	13.71 ± 4.42	–	–
	Isomaltose	266.25 ± 234.83	–	1973.48 ± 1279.07	82.50 ± 18.52	446.49 ± 507.92	23.10 ± 14.69
	Lactitol	58.34 ± 29.45	5.78 ± 1.61	–	–	–	–
	Lactobionic acid	–	13.49 ± 5.76	–	–	–	41.63 ± 17.21
	Lactose	–	–	287.10 ± 215.74	116.90 ± 27.56	365.94 ± 357.66	–
	Lactulose	287.73 ± 234.22	–	691.53 ± 562.01	34.38 ± 19.63	–	–
	Leucrose	–	–	60.79 ± 35.88	58.55 ± 43.42	–	0.74 ± 0.43
	Maltitol	33.51 ± 23.36	18.76 ± 17.92	–	–	–	–
	Maltose	327.70 ± 152.04	13.58 ± 9.49	86.63 ± 60.29	69.47 ± 53.76	341.04 ± 307.75	81.90 ± 71.00
	Maltotriitol	–	5.99 ± 3.06	–	–	–	9.25 ± 7.72
	Maltotriose	24.22 ± 28.75	0.43 ± 0.47	–	1.17 ± 0.75	3.11 ± 3.64	–
	Melezitose	47.68 ± 52.35	8.66 ± 16.52	306.10 ± 184.55	36.23 ± 11.64	–	8.06 ± 4.17
	Melibiose	326.79 ± 255.96	9.31 ± 2.75	2282.93 ± 1547.85	78.06 ± 38.37	820.50 ± 1300.72	48.83 ± 38.15
	Methyl-beta-D-galactopyranoside	93.76 ± 62.52	–	15.87 ± 8.84	30.22 ± 40.73	54.09 ± 67.02	8.35 ± 6.10
	Mucic acid	17.32 ± 8.98	4.88 ± 0.0001	–	11.34 ± 3.24	–	–
	Myo-inositol	368.08 ± 241.42	93.27 ± 0.0001	–	2507.23 ± 928.08	–	187.93 ± 141.25
	Phenyl-beta-glucopyranoside	–	–	131.47 ± 103.71	–	–	–
	Raffinose	152.56 ± 86.40	33.83 ± 1528.92	1888.72 ± 1987.89	64.78 ± 16.64	591.54 ± 749.26	218.52 ± 78.38
	Ribonic acid-gamma-lactone	11.91 ± 6.15	1.38 ± 18.93	50.14 ± 72.54	–	8.25 ± 6.89	–
	Sophorose	28.61 ± 17.09	5.53 ± 35.25	267.57 ± 397.84	101.31 ± 53.55	–	–
	Sucrose	35.49 ± 22.44	93.28 ± 2977.37	3113.89 ± 2600.48	3709.27 ± 2144.85	202.51 ± 149.82	29.92 ± 12.26
	Tagatose	43.29 ± 35.02	21.29 ± 992.69	582.56 ± 489.04	506.86 ± 380.09	168.24 ± 152.90	95.02 ± 74.39
	Talose	–	29.66 ± 696.90	231.51 ± 316.25	–	36.43 ± 45.94	–
	Turanose	–	–	–	282.84 ± 67.57	–	5.93 ± 2.61
Alkaloid	Piperine	–	199.76 ± 39.09	–	33.65 ± 7.67	–	–
Others	Adenine	–	12.97 ± 0.0001	–	–	–	–
	Adenosine	–	–	–	–	–	11.84 ± 4.49
	Guanosine	8.80 ± 9.51	–	–	–	–	–
	Hypoxanthine	–	0.23 ± 0.0001	–	–	–	–
	Lactamide	–	0.19 ± 0.0001	–	–	–	–
	Nicotinic acid	10.03 ± 6.12	1.14 ± 0.13	14.58 ± 11.14	9.47 ± 3.71	6.13 ± 4.88	1.65 ± 0.67
	Nicotinamide	–	–	–	–	–	0.06 ± 0.07
	2-Piperidone	–	0.28 ± 0.0001	–	–	–	–
	Porphine	18.33 ± 8.65	2.80 ± 26.45	19.62 ± 14.48	44.66 ± 46.01	6.29 ± 3.42	13.33 ± 4.12
	Thymine	0.31 ± 0.17	0.21 ± 1.04	0.79 ± 0.22	–	0.13 ± 0.10	0.33 ± 0.21
	Uracil	0.77 ± 0.64	0.07 ± 1.61	110.56 ± 219.41	2.65 ± 1.53	160.15 ± 237.11	4.54 ± 8.38
	Urea	–	0.29 ± 0.0001	–	–	–	0.56 ± 0.51

– denotes not detected

inhibit acetylcholinesterase enzymes and showed selective inhibition of the AChE enzyme with the inhibitor constant (K_i) = 1.5–18.9 μ M (Friggeri et al. 2015).

In our study, isocitric acid was also detected in the leaves of *P. nigrum* and Tynpew variety of *P. betle*, but in lesser concentration than fruits of *P. longum* samples. Tartaric

acid was observed in leaves of *P. retrofractum* in highest content, but also determined in lower content in leaves of *P. nigrum* and stem extract of *P. retrofractum*. Maltitol, lactitol, 4-guanidinobutyric acid, L-asparagine, 3,4-dihydroxybenzoic acid, pyruvic acid and maltotriose were detected in leaves and stems of *P. retrofractum* in high content (Fig. 3).

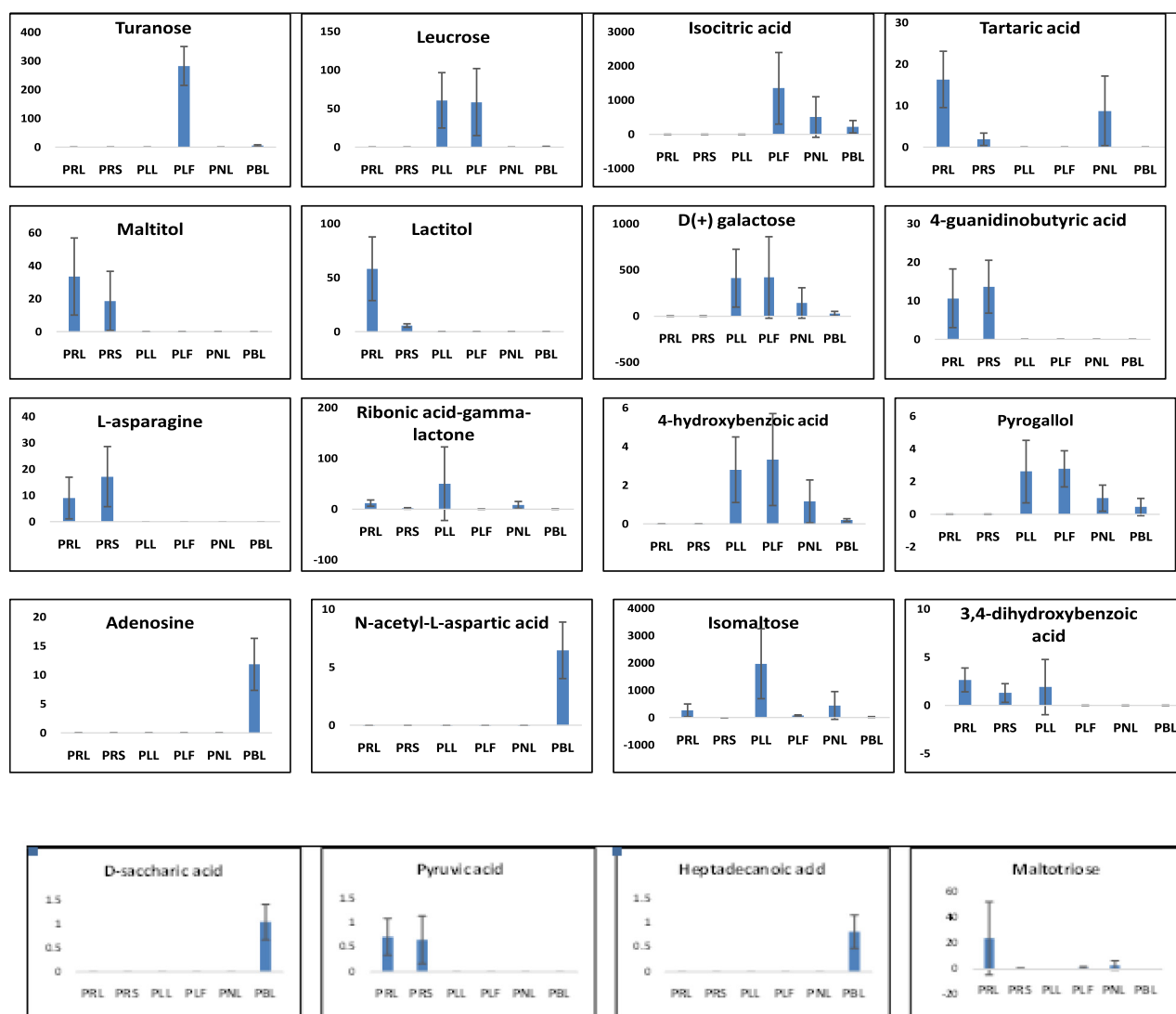


Fig. 3 Differences in amount of key contributory metabolites (based on overall coefficient scores) as identified from PLS-DA. The error bars are the standard deviations from the four biological replicates.

After negative correlation analysis between the IC_{50} values of different enzyme inhibition assays and the detected metabolites via GC/MS analysis, and on the basis of availability in our laboratory we determined a few metabolites, viz., piperine (alkaloid), 1,2,4-benzene triol, 3,4-dihydroxy benzoic acid, benzoic acid, gallic acid, ferulic acid, quinic acid (all grouped to phenolic compounds), sugars like D-glucose, D-(+) melezitose and amino acid like glycine and were further assayed against acetylcholinesterase, α -amylase and α -glucosidase enzymes and heam biocrystallization (Tables 4 and 5) and compared with the standard drugs. Among these, piperine showed higher anti-cholinesterase activity, with an IC_{50} value of $0.837 \pm 0.012 \mu M$, which is quite lower than the IC_{50} value ($1.42 \pm 0.02 \mu M$) of standard drug Physostigmine. Piperine also showed its

PRL=*Piper retrofractum* leaf, PRS=*P. retrofractum* stem, PLL=*P. longum* leaf, PLF=*P. longum* fruit, PNL=*P. nigrum* leaf, PBL=*P. betle* leaf

inhibitory activity against α -glucosidase enzyme but higher IC_{50} than the standard drug (Acarbose). Based on the study of Manap et al. (2019), between curcumin and piperine, piperine showed stronger binding affinity (-10.5 kcal/mol) and greater interaction with AChE with an IC_{50} value of $76.6 \pm 0.08 \mu g/mL$. In this study curcumin and piperine combination showed synergistic affinity towards AChE and simultaneous interactions with the key amino acids of catalytic action sites (CAS) and peripheral anionic site (PAS) of AChE. 1,2,4 benzene triol inhibited all the three studied enzymes but showed lesser activities than their corresponding standard drugs (Table 4), but 1,2,4 benzene triol showed very high activity against heme biocrystallization assay than the standard drug chloroquine diphosphate (Table 5). Das et al. (2016) and Debnath et al. (2024) from



Table 7 Molecular docking studies of Acetylcholinesterase

Sl. no.	Protein (acetylcholinesterase)	Ligand	Free energy ΔG (kcal/ mol)	Number of hydrogen bonds
1.	<i>Electrophorus electricus</i> acetylcholinesterase (PDB ID: 1C2O)	Donepezil	− 8.7	–
2.		Physostigmine	− 7.4	–
3.		1,2,4-Benzenetriol	− 5.7	Glu313, Asn233, His405
4.		3,4-Dihydroxy benzoic acid	− 6.2	Asn233, Glu313, His405
5.	Binary complex of native human Acetylcholinesterase with Donepezil (PDB ID: 6O4W)	Donepezil	− 8.3	–
6.		Physostigmine	− 8.5	–
7.		1,2,4-Benzenetriol	− 5.6	Glu202
8.		3,4-Dihydroxy benzoic acid	− 6.5	Tyr133, Tyr337

Table 8 Molecular docking studies of alpha-amylase

Sl. no.	Protein	Ligand	Free energy ΔG (kcal/mol)	Amino acids involved in hydrogen bonding
1.	Porcine pancreatic alpha-amylase (PDB ID: 1PIF)	Acarbose	− 7.4	Trp58, Asp197, His201, Arg 195, Glu233
2.		1,2,4-Benzenetriol	− 5.4	Ser310, Arg267, Ile312
3.		Benzoic acid	− 5.3	Arg195
4.		Gallic acid	− 6.1	Ile312, Thr314
5.	Human pancreatic alpha-amylase (PDB ID: 1HNY)	Acarbose	− 7.6	Thr6, Ser226, Arg252, Asp402, Gly403,
6.		1,2,4-Benzenetriol	− 5.1	Ala128, Glu181
7.		Benzoic acid	− 5.3	Glu233, His299, Arg195
8.		Gallic acid	− 5.8	Tyr182, Lys178, Glu181, Val129

our laboratory previously reported the bio efficacy of benzene 1,2,4-triol against α -amylase, α -glucosidase and against heam biocrystallization for the first time. The presence of this metabolite in the chosen *Piper* species further validate these bioactivities. 3,4-dihydroxy benzoic acid showed its activity only against AChE, whereas benzoic acid and gallic acid showed its inhibition against alpha amylase only.

Gallic acid also showed very high anti-malarial activity much lower IC_{50} value than the standard drug compound. Friggeri et al. (2015) demonstrated the selective activity of 3,4-dihydroxy benzoic acid derivatives to inhibit AChE with $K_i = 1.5\text{--}18.9 \mu\text{M}$. According to the study done by Guan et al. (2022), 2,3,4-trihydroxy benzoic acid had very strong inhibition against α -amylase ($IC_{50} = 17.30 \pm 0.73 \mu\text{M}$).

Table 9 Molecular docking studies of α -glucosidase

Sl. no.	Protein	Ligand	Free energy ΔG (kcal/ mol)	Number of hydrogen bonds
1.	Crystal structure of isomaltase from <i>Saccharomyces cerevisiae</i> (PDB ID: 3A4A)	1,2,4-Benzenetriol	− 5.7	Asn235, Asn317
2.		Ferulic acid	− 4.8	Leu394, Glu477
3.		Quinic acid	− 6.5	Lys156, Asn235, His423
4.		D-(+) melezitose	− 6.1	Arg359, Thr358, Phe321, Lys523, Asp362
5.		Acarbose	− 6.9	Thr358
6.	Crystal structure of human lysosomal acid-alpha-glucosidase, GAA, in complex with acarbose (PDB ID: 5NN8)	1,2,4-Benzenetriol	− 5.7	Asp404, Arg600, His674
7.		Ferulic acid	− 6.0	Arg608, Val867, Ser864
8.		Quinic acid	− 5.7	His584, Leu868, Val867, His717
9.		D-(+) melezitose	− 6.8	Tyr360, Arg608, Met363, His717
10.		Acarbose	− 7.2	Arg189, Arg190, Lys162, Asn188, Asp163

Gallic acid is more or less ubiquitously present as plant food components. Oboh et al. (2016) revealed that combination of acarbose and gallic acid (1:1) and acarbose and gallic acid (3:1) showed highest α -amylase and α -glucosidase inhibition effect respectively. In our study gallic acid did not show any α -glucosidase and AChE inhibition. Ferulic acid, quinic acid, D-glucose, D-(+) melezitose and glycine were also assessed but they only displayed their inhibition against α -glucosidase enzyme (Tables 4 and 6). Using molecular docking and virtual screening ferulic acid showed significant inhibition against α -glucosidase enzyme (Rasouli et al. 2017) also validate our result. Enzyme kinetics, fluorescence spectra and molecular docking analyses performed by Han et al. (2024) indicated that quinic acid is a potent α -glucosidase inhibitor with an IC_{50} of 5.46 mM, also validate our analysis. According to our study, the anti-diabetic potentiality by inhibiting α -glucosidase via glycine, D-(+)

melezitose and D-glucose has not been reported by any worker earlier.

Molecular docking analysis

Molecular docking studies of the phytochemicals against the said enzymes were performed by AutoDockVina. The binding energies of all the ligand–protein interactions were observed to be having significant negative free energies implying positive interaction. However, strength of their interactions varied significantly amongst each set of ligand and protein (Tables 7, 8 and 9). This is due to variable interactions of ligands with surrounding amino acids with diverse bonds including hydrogen bonding, Vander Waal interaction, hydrophobic interaction, Pi-Pi, Pi-anion, and Pi-cation interactions (Figs. 4, 5 and 6).



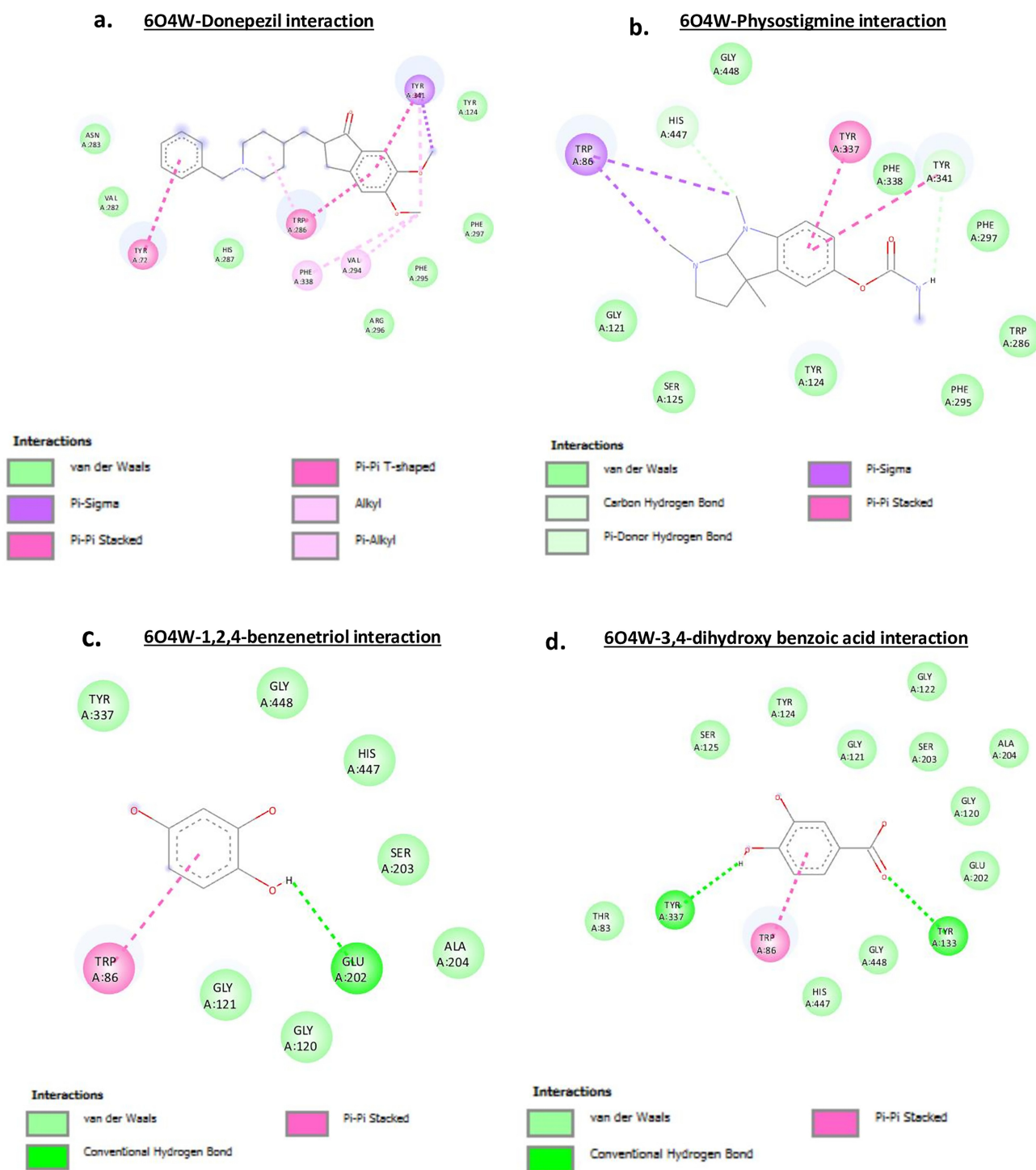


Fig. 4 2-D structures of Acetylcholine-ligand interaction with **a** donepezil, **b** physostigmine, **c** 1,2,4-benzenetriol, **d** 3,4-dihydroxy benzoic acid

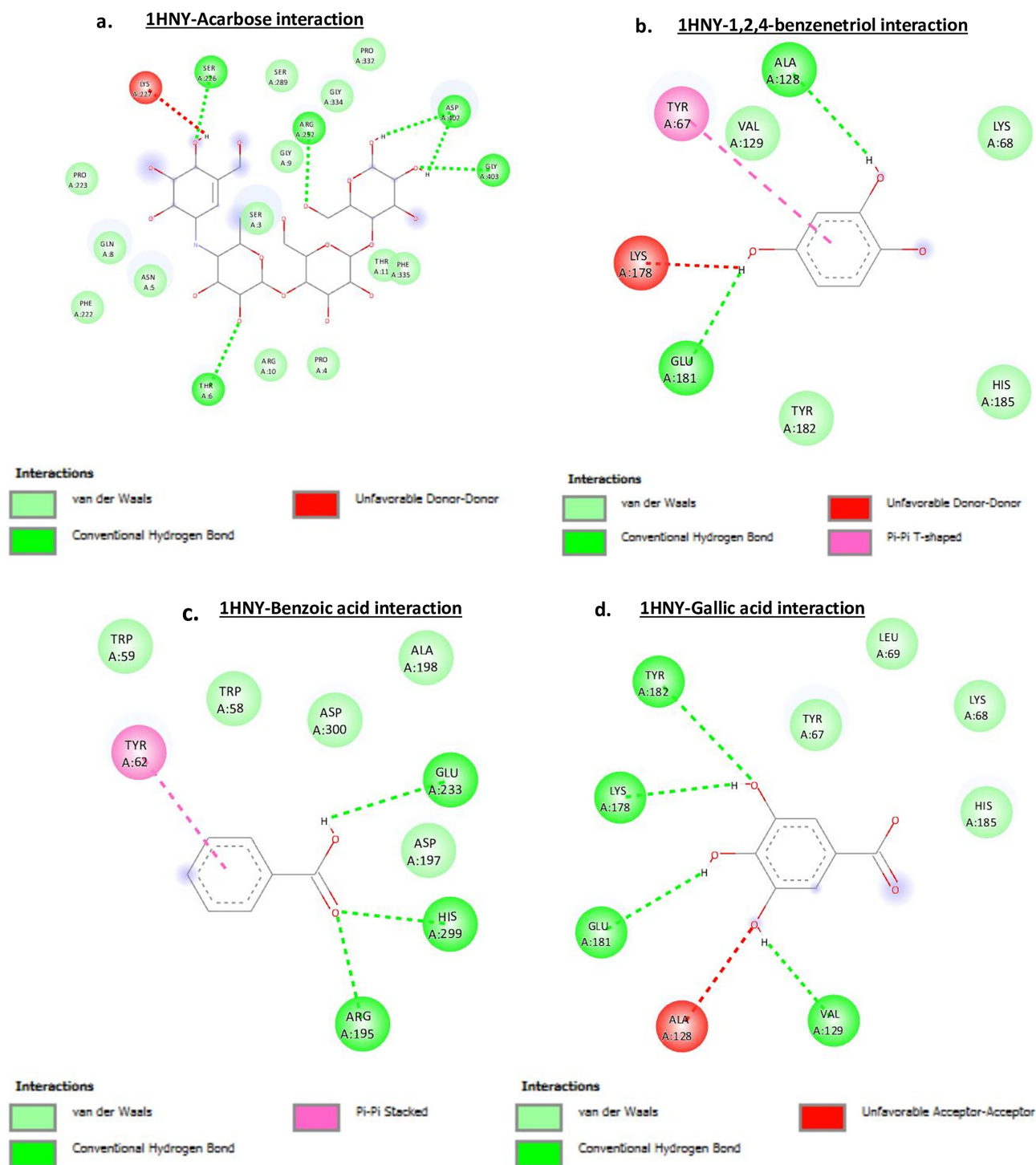


Fig. 5 2-D structures of α -amylase-ligand interaction with **a** acarbose, **b** 1,2,4-benzenetriol, **c** benzoic acid, **d** gallic acid

Conclusion

In summary, all the extracts showed significant anti-cholinesterase, anti-glycosidase, anti-malarial and antioxidant activities in a dose dependant manner. The bioactivities were compared with the standard drugs and in silico analysis

established their efficacy as an alternative to synthetic drugs. Several metabolites viz., 1,2,4 –benzene triol, 3,4-dihydroxy benzoic acid, gallic acid, benzoic acid, ferulic acid, quinic acid, D(+)-melezitose and piperine detected showed strong biological activities.



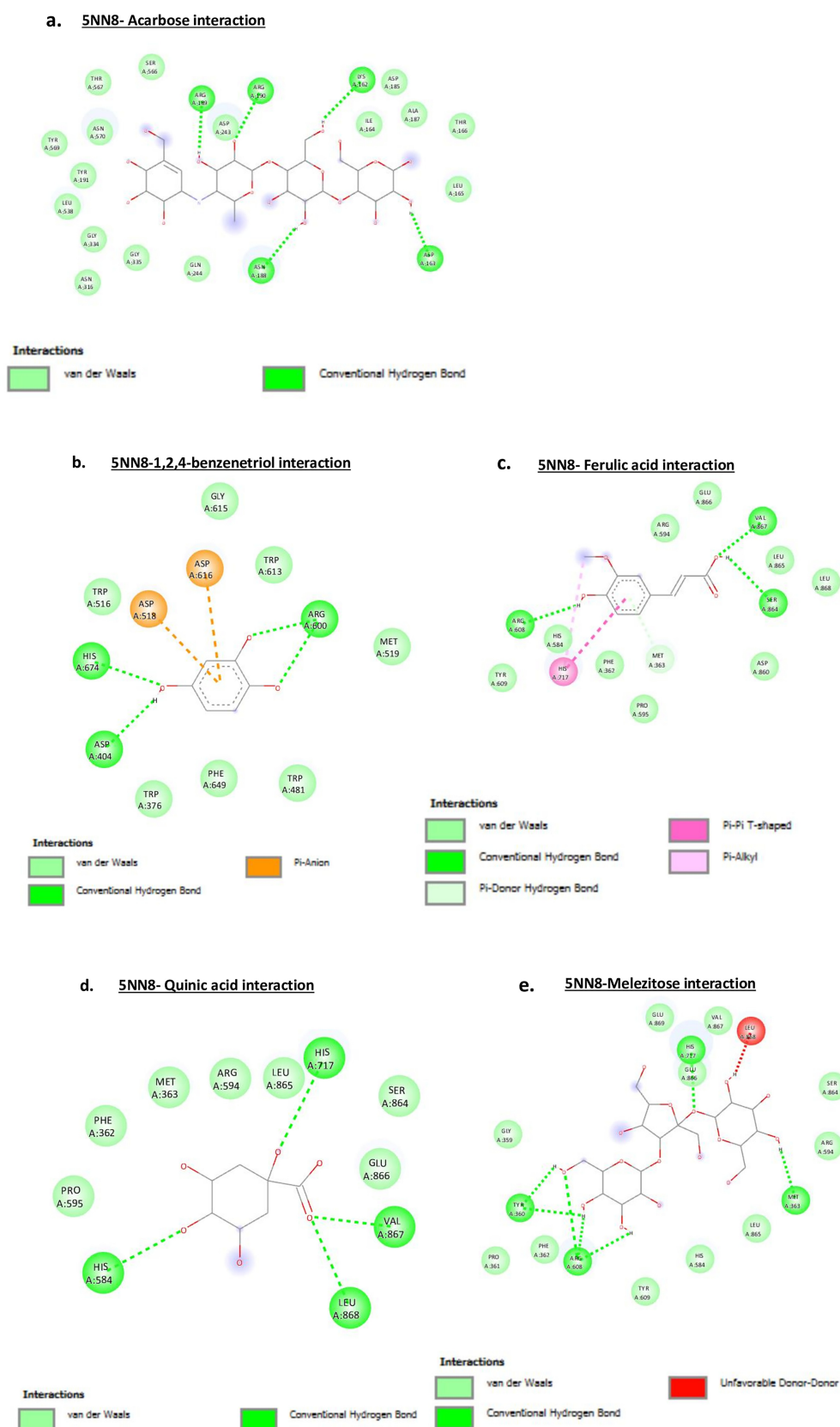


Fig. 6 2-D structures of α -glucosidase-ligand interaction with **a** acarbose, **b** 1,2,4-benzenetriol, **c** ferulic acid, **d** quinic acid, **e** melezitose

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Data availability Not applicable.

Declarations

Conflict of interest The authors declare that they do not have any conflict of interest.

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