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In vitro anti-diabetic potential of medicinal herbs commonly used in the Ayurvedic system of Sri Lanka with comprehensive metabolite profiling of *Phyllanthus emblica* using GC-MS and LC-HRMS

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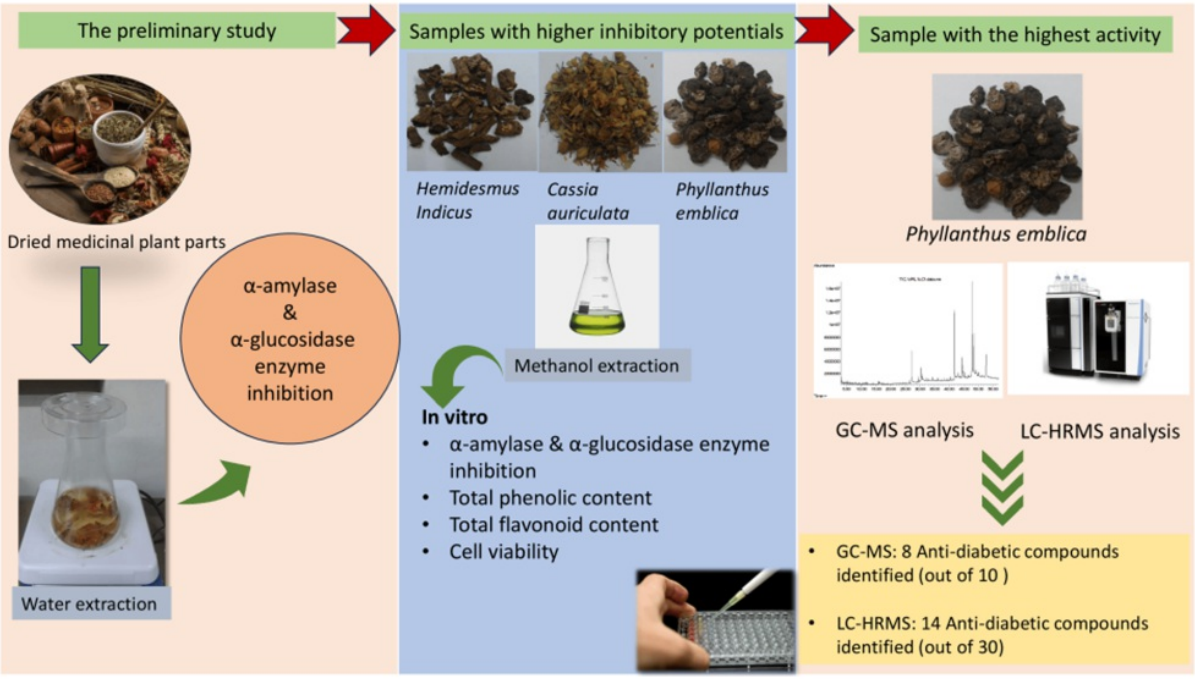
ABSTRACT

This study assessed the *in vitro* anti-diabetic potential and bioactive constituents of ten Sri Lankan medicinal herbs. Initial screening of aqueous extracts for starch-digesting enzyme inhibition prioritised three plants with notable activity ($p \leq 0.05$), for further assessment using methanolic extracts: *Phyllanthus emblica* (PE), *Cassia auriculata* (CA), and *Hemidesmus indicus* (HI). The selected plants were tested for starch-digesting enzyme inhibition, cytotoxicity, and bioactive metabolite identification, with PE subjected to GC-MS and LC-HRMS analyses. All three extracts contained alkaloids, flavonoids, tannins, and terpenoids, except saponins and steroids in PE. GC-MS analysis of PE annotated ten compounds, eight with anti-diabetic properties, while LC-HRMS annotated thirty metabolites, including fourteen anti-diabetic compounds. Cell viability assessments confirmed the non-toxic nature of PE, CA, and HI. The significant enzyme inhibition and non-toxic nature of PE highlight its potential to treat type 2 diabetes. Further *in vivo* and clinical studies are essential to determining effective dosage and toxicity levels.

Graphical Abstract



KEYWORDS

Medicinal plants; anti-diabetic; starch-digesting enzyme; GC-MS; LC-HRMS

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Introduction

Diabetes Mellitus (DM) is a prominent metabolic disorder, with the International Diabetes Federation (IDF) estimating 536.6 million cases in 2021, projected to rise to 783 million by 2045. There are primarily two types of DM: type 1 (T1DM) and type 2 DM (T2DM). T2DM accounts for over 90% of DM cases and primarily arises from insulin resistance or abnormal insulin secretion (Patel et al. 2012; Nazarian-Samani et al. 2018; Hasanpour et al. 2020; Ogurtsova et al. 2022).

Managing T2DM involves reducing postprandial hyperglycaemia (PPHG) by inhibiting the enzymatic activities of α-amylase and α-glucosidase, responsible for carbohydrate digestion and glucose absorption in the digestive tract, respectively. Due to the various side effects of synthetic medicines, medicinal plants are gaining growing attention worldwide. The World Health Organisation (WHO) reports that nearly 80% of the population in many developing countries relies on alternative medicine derived from medicinal plants (Krishnaraju et al. 2005; Tugume and Nyakoojo 2019; Okaiyeto et al. 2023).

Sri Lanka, a tropical island abundant in numerous medicinal plants, lacks scientific validation for the functional properties and therapeutic applicability of most of these plants. Therefore, this study aims to assess the *in vitro* anti-diabetic potential of ten medicinal herbs commonly used in the Ayurvedic system of Sri Lanka. The plants demonstrating the highest activity would undergo further evaluation to identify their lead bioactive constituents responsible for treating T2DM. The findings of this study may not only contribute to scientifically validating the anti-diabetic properties of some Ayurvedic plants but also help identify novel compounds for managing T2DM or its associated complications.

Results and discussion

α-Amylase and α-glucosidase inhibitory activity

α-Amylase and α-glucosidase inhibitory activities of selected plant samples are listed in Table 1. As the preliminary study for the current investigation, we assessed the inhibitory potential of α-amylase and α-glucosidase in aqueous extracts from ten commonly consumed medicinal herbs in Sri Lanka (Table 1). Many individuals in Sri Lanka regularly drink or use these particular plants as herbal tea to meet their daily healthcare needs or as a beverage. Extracts from *Phyllanthus emblica* (PE), *Cassia auriculata* (CA), and *Hemidesmus indicus* (HI) that showed significantly higher inhibitory potentials ($p \leq 0.05$) were further assessed using methanol extraction to determine their effectiveness in inhibiting starch-digesting enzymes. The findings suggested that, in comparison to the activity of water extracts, the methanol extracts exhibited a significantly stronger inhibitory effect ($p \leq 0.05$) against both α-amylase and α-glucosidase enzymes among all three examined extracts (Table 1).

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Table 1. α-Amylase and α-glucosidase inhibitory activities of plant parts in water and methanol extracts.

No	Medicinal plants	Parts of plant	Water Extract (IC_{50} µg/mL)		Methanol Extract (IC_{50} µg/mL)	
			α-Amylase inhibitory activity	α- Glucosidase inhibitory activity	α-Amylase inhibitory activity	α- Glucosidase inhibitory activity
1	*P. emblica*	Fruit	40 ± 1.50^{i}	310 ± 5.7^{c}	3.14 ± 0.30	1.48 ± 0.05
2	*C. auriculata*	Flower	730 ± 7.70^{h}	1870 ± 10^{b}	5.12 ± 1.34	1.71 ± 0.10
3	*H. indicus*	Root	4200 ± 140^{g}	4900 ± 190^{a}	36.87 ± 0.90	8.51 ± 0.10
4	*A. marmelos*	Fruit	7400 ± 3.53^{c}	20% (10^{5} µg/mL)	–	–
5	*A. marmelos*	Flower	5900 ± 14.14^{e}	35.85% (10^{5} µg/mL)	–	–
6	*T. cordifolia*	Stem	8790 ± 220^{b}	15.51% (10^{5} µg/mL)	–	–
7	*C. fenestratum*	Stem	5330 ± 117.5^{f}	8.16% (10^{5} µg/mL)	–	–
8	*A. lanata*	Aerial parts	6350 ± 250^{d}	3.43% (10^{5} µg/mL)	–	–
9	*S. rhombifolia*	Aerial parts	9760 ± 530^{a}	2.31% (10^{5} µg/mL)	–	–
10	*S. dulcis*	Aerial parts	7460 ± 250^{c}	90.67% (10^{5} µg/mL)	–	–
	Acarbose		5.57 ± 4.30			

*Data are presented as mean ± standard deviation (n = 3). Mean values with different superscript letters in a column are significantly different ($p \leq 0.05$).

The management of type 2 diabetes mellitus (T2DM) involves delaying the enzyme activities of α-amylase and α-glucosidase, which are responsible for the [replace the sentence section highlighted to 'the digestion of carbohydrates and the absorption of glucose in the digestive tract '] carbohydrate digestion and absorption of glucose in the digestive tract (Ighodaro et al. 2016). The α-amylase enzyme breaks down complex polysaccharides into oligosaccharides and disaccharides, which are then hydrolysed by the α-glycosidase enzyme into simple absorbable monosaccharides (Sapkota et al. 2022). The inhibition of both α-amylase and α-glycosidase delays carbohydrate digestion and subsequent

glucose absorption, thereby lowering postprandial glucose levels (Ighodaro et al. 2016; Sapkota et al. 2022). Numerous studies have explored the potential of medicinal plants in treating T2DM (Jarald et al. 2008; Patel et al. 2012; Sapkota et al. 2022). However, due to insufficient and sustained scientific evidence, a considerable number of medicinal plants have not achieved prominence as effective medicines.

According to the findings of the present study, for [replace the word 'for' with 'in'] the α-amylase inhibitory assay, water extracts exhibited IC_{50} values varied from 40 ± 1.50 to 9760 ± 530 µg/mL, while in methanol all three samples showed over 90% inhibition at 308 µg/mL, with IC_{50} ranging from 3.14 ± 0.30 to 36.87 ± 0.90 µg/mL (Table 1). IC_{50} value represents the concentration of the extract that causes 50% inhibition of enzyme activity. Consequently, higher inhibitory potentials are linked to smaller IC_{50} values. For the α-glucosidase inhibitory assay, W [simple case 'w']ater extracts of PE, CA, and HI showed IC_{50} values ranging from 310 ± 5.7 to 4900 ± 190 µg/mL. All other samples showed less than 50% inhibition at 10^5 µg/mL concentration. Methanol extracts of PE, CA, and HI exhibited IC_{50} ranging from 1.48 ± 0.05 to 8.51 ± 0.10 µg/mL. According to the statistical analysis, PE demonstrated significantly the highest inhibitory potential ($p \leq 0.05$) for both starch-digesting enzyme inhibitory assays, followed by CA and HI, based on the results of both water and methanol extracts.

Numerous studies have documented the efficacy of these three extracts in managing T2DM (Das and Singh Bisht 2013; Nille et al. 2021; Sapkota et al. 2022). In a previous study by Shrestha et al. (2021), the methanol extract of PE exhibited potent α-amylase inhibitory activity (using the DNSA assay method) and α-glucosidase inhibitory activity, yielding IC_{50} values of 397.67 µg/mL and 0.48 µg/mL, respectively. Upon comparing the α-amylase inhibitory activity of PE in our study with the reference study, there was an approximately 100-fold difference in the IC_{50} values. This notable contrast may be due to the interference in the DNSA method utilised in the reference study and environmental factors.

Acarbose is a pseudo tetrasaccharide that suppresses the [remove 'the'] α-amylase activity by competitive and reversible inhibition (Yilmazer-Musa et al. 2012). All plant extracts tested in our study, except the methanol extract of PE, demonstrated lower inhibitory potential when compared to commercially available acarbose, which served as the positive control in this study.

The findings of this study provide promising insight into the potential of utilising extracts of PE, CA, and HI as therapeutic agents for treating T2DM. However, the complexity of the human digestive system, including interactions with other dietary components, and enzymatic activities in the gastrointestinal tract, may significantly influence the bioavailability of bioactive compounds present in these extracts when consumed. Therefore, further studies, including *in vivo* and human intervention studies, are essential to fully understand the therapeutic potential of these extracts in managing T2DM.

The crude methanol extracts of PE, CA, and HI were again partitioned with solvents of increasing polarity using n-hexane, dichloromethane (DCM), and ethyl acetate (EtOAc) to assess α-amylase and α-glucosidase enzyme inhibitory potentials at a concentration of 77 µg/mL (Supplemental Table S1). The results demonstrated that both enzymes were notably inhibited in all three solvent fractions of PE. The HI extracts showed significant inhibition solely in the EtOAc fraction. In contrast, the CA demonstrated over 90% α-amylase inhibition in the EtOAc fraction and α-glucosidase inhibition in both DCM and EtOAc fractions (Supplemental Table S1). Therefore, both nonpolar and polar compounds could be responsible for the enzyme inhibition of PE, and only moderate to high polar compounds may be responsible for the enzyme inhibition of CA, and HI. Our findings resemble with the previous study showing significant inhibitory activity in various fractions of dried fruit of *P. emblica* (Sapkota et al. 2022).

Phytochemical screening

Phytochemical screening of PE, CA, and HI indicated that alkaloids, flavonoids, tannins, and terpenoids were present in all extracts, while saponins and steroids were absent in the PE extract (Supplemental Table S2).

Total phenolic content (TPC)

Phenolic concentrations in both aqueous and methanol plant extracts varied widely, ranging from (7.82 ± 0.22 to 271.23 ± 4.06 mg GAE/g for water extracts and 680 ± 14.142 to 2100 ± 141.42 mg GAE/g for methanol extracts), as shown in Supplemental Figure S2. According to the statistical analysis ($p \leq 0.05$) the highest TPC was recorded in PE followed by CA ($p \leq 0.05$) in both extracts.

Total flavonoid content (TFC)

Flavonoid content in the plant extracts varied widely, ranging from 1.86 ± 0.08 to 85.51 ± 0.43 mg CE/g dry weight in water extracts and from 61.8 ± 4.24 to 265.33 ± 2.83 mg CE/g dry weight in methanol. PE reported the maximum TFC ($p \leq 0.05$) in water extract, followed by the CA. In contrast, PE had the least activity, while HI reported the highest TFC in methanol extracts (Supplemental Figure S3).

GC-MS profiling of hexane extract

In this study, the hexane fraction of PE exhibited strong inhibitory activity against both α-amylase and α-glucosidase enzymes (Supplemental Table S1). Consequently, GC-MS analysis was conducted to annotate the volatile organic compounds responsible for its anti-diabetic effect (Supplemental Figure S4, Table S3).

The GC-MS method is extensively used in plant metabolite profiling due to its high sensitivity and ability to offer the high resolution required to separate the principal compounds from plant extracts (Rokkam et al. 2022). However, to the best of our knowledge, no study has been reported on GC-MS-based metabolite profiling to detect bioactive compounds in n-hexane extracts of dried fruits of PE.

In the current study, GC-MS analysis tentatively identified ten bioactive compounds, including terpenes (**7, 8, 9, 10**), steroids (**5** and **6**), monounsaturated long-chain fatty acids (**3** and **4**), saturated long-chain fatty acids (**2**), and fatty acid methyl ester (**1**). Remarkably, eight compounds such as terpenes (**7, 8, 9, 10**), steroids (**5** and **6**), and fatty acids (**2** and **4**) were previously reported for their anti-diabetic properties through a variety of mechanisms including *in vitro* inhibition of α-amylase, β-glucosidase and protein tyrosine phosphatase-1B (PTP1B) enzyme activities, as well as lowering serum glucose levels in experimental animal models (Narender et al. 2009; Gupta et al.

2011; Siddique and Saleem 2011; Ko et al. 2016; Ngenge Tamfu et al. 2022).

Furthermore, a prior study done by Su et al. (2013), found that compounds **2** and **4** exhibited a delayed mechanism of carbohydrate digestion by inhibiting α-amylase enzyme activity, with IC_{50} values of 0.16 mg/mL and 0.0272 mg/mL, respectively. Additionally, the α-amylase inhibitory activity of steroids, particularly compounds **6** and terpenes **(7** and, [remove comma] **9**) has been previously reported, showing varying IC_{50} values of 2939 ± 76 µM, 248 ± 12 µM, and 2585 ± 79 µM respectively (Yuca et al. 2022). Moreover, in another study, (Javed et al. 2022), terpenes, particularly compounds **9, 7, 6,** and **10,** were reported to delay sugar absorption by interacting with the α-glucosidase enzyme, inhibiting its ability to break down complex carbohydrates into glucose. Their IC_{50} values ranged from 15.87 ± 1.16 µM, 21.49 ± 0.51 µM, 65.31 ± 0.96 µM and 18.14 ± 0.27 µM respectively, and these compounds displayed significant inhibitory potential compared to acarbose (IC_{50} of 38.25 ± 1.12 µM), except for compound **6** in this study. Notably, most of these compounds have been previously reported for their potent anti-diabetic activity with promising α-amylase and α-glucosidase inhibitory activity, suggesting that they may have contributed to the significantly higher anti-diabetic effect observed in PE in the present study.

LC-HRMS profiling of dichloromethane and ethyl acetate fractions

DCM and EtOAc subfractions of PE were further analysed using LC-HRMS to annotate the bioactive constituents responsible for their anti-diabetic activity (Supplemental Table S4). Results revealed that, among the tentatively identified compounds, eighteen were detected in the DCM extract, whereas the EtOAc extract contained twelve compounds. These compounds can be categorised into various subclasses, including terpenes (**11, 18, 22, 24, 26**, and **40**), quinones (**12, 13**, and **17**), xanthones (**14** and **34**), alkaloids (**16** and **38**), fatty acids (**20** and **28**), flavonoids (**21**, **36**, and **39**), phenols (**29, 30, 32**, and **33**), and tannins (**12** and **15**) (Supplemental Table S4).

Among these, only fourteen compounds (**13, 14, 15, 18, 21, 23, 26, 29, 30, 32, 33, 34, 36**, and **38**) were previously recognised for their anti-diabetic properties using ranged of mechanisms, including *in vitro* inhibition of enzyme activities such as protein tyrosine phosphatase-1B (PTP1B), dipeptidyl

peptidase-4 (DPP-IV), α-amylase, α-glucosidase, and more (Cui et al. 2007; Bu et al. 2010; Ibrahim et al. 2019; Rakotondrabe et al. 2022).

A prior study demonstrated that compound **5**, extracted from *Laminaria japonica*, exhibited significant α-glucosidase inhibitory activity with an IC_{50} value of 38 μM (Bu et al. 2010). Additionally, recent research (Kovalenko et al. 2020) utilised compound **18** to create active pharmaceutical ingredients for treating T2DM by combining it with an organic compound of *o*-phenylenediamine. Further, a previous study (Cui et al. 2007) highlighted the potential of compound **21** within the flavonoid group for treating T2DM through *in vitro* PTP1B inhibitory activity. Another study (Liu et al. 2021) reported the substantial contribution of compounds **29** and **30** to the mechanism of delayed sugar absorption through α-glucosidase enzyme inhibition. Notably, compound **34** exhibited a remarkable amylase inhibitory activity with an 81.8% inhibition percentage (Ibrahim et al. 2019). Furthermore, the α-glucosidase inhibitory effect of compound **36** was reported as an IC_{50} value of 42.47 ± 4.13 μM, which was much better than that of the activity of positive drug acarbose (109.54 ± 14.23 μM) (Rakotondrabe et al. 2022). The collective evidence suggests that the PE extract contains a variety of bioactive constituents, some of which possess well-documented anti-diabetic properties, indicating its potential significance and utility as a supplement for managing T2DM.

Cell viability assay

Cell viability assay was employed to enhance the reliability of our findings and to investigate their therapeutic potential. Total extracts and their fractions of PE, CA, and HI were tested against HeLa (cervix adenocarcinoma) cell line, and results are shown in (Supplemental Table S5). Hexane, DCM, and EtOAc extracts of PE did not exhibit any toxicity towards HeLa cells. Similarly, hexane and DCM extracts of CA, and hexane extract of HI were not toxic to HeLa cells. However, other extracts of CA and HI showed moderate toxicity at the same starting concentration of 50 μg/mL (Supplemental Table S5).

Our findings are consistent with previous studies that demonstrated the low or no toxicity of *P. emblica* fruit extracts on various cell lines, including HeLa cells (IC_{50}- 30.45 μg/mL) and MRC5 cells (IC_{50} > 400 μg/mL) (Ngamkitidechakul et al. 2010; Dinesh et al. 2017). Similarly, (Kalaivani and

Uava 2008 [This intext citation is not linked to the reference list.]) found that the administration of ethanol extract of *C. auriculata* flowers up to a dosage of 1000 mg/kg body weight/day for a month resulted in no toxicity signs on male Wistar albino rats. In the case of *H. indicus*, (Shilpha et al. 2022) reported that the roots of *H. indicus* had a low cytotoxicity effect on human embryonic kidney cell lines (HEK-293), with 81.6% cell viability observed at a concentration of up to 300 µg/mL.

Conclusion

In the present study, *Phyllanthus emblica* (PE) exhibited the highest inhibitory potential ($p \leq 0.05$) for both the α-amylase and α-glucosidase inhibitory assay methods, followed by *Cassia auriculata* (CA), and *Hemidesmus indicus* (HI) in both water and methanol extracts. Both nonpolar and polar compounds could be responsible for the anti-diabetic activity of PE, while only moderate to high polar compounds may be responsible for the anti-diabetic activity of CA and HI. Phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, and terpenoids in all three extracts, excluding saponins and steroids in PE. GC-MS analysis of the n-hexane fraction of PE identified ten compounds, eight of which were previously recognised for their anti-diabetic properties. LC-HRMS analysis of dichloromethane (DCM) and ethyl acetate (EtOAc) subfractions of PE revealed the presence of 30 [Please type the number 30 as "thirty".] metabolites, with 14 [Please type the number 14 as "fourteen".] compounds known for their anti-diabetic activities. Cell viability assessments demonstrated the non-toxic nature of hexane, DCM, and EtOAc extracts of PE. Similarly, hexane and DCM extracts of CA and hexane extract of HI showed no toxicity, while other extracts of CA and HI exhibited moderate toxicity at a starting concentration of 50 mg/mL for HeLa cells. The significant anti-diabetic properties of PE and its non-toxic characteristics, underscore its potential to develop nutraceuticals to treat T2DM. Further *in vivo* and clinical studies are necessary to determine the effective doses and toxicity levels, as excessive consumption may pose health risks.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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