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Research Article

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A Comprehensive Evaluation of the Anti-Hyperglycemic Activity of *Callistemon citrinus* (Curt.): HPLC Fingerprint Analysis and Its Association with the Biological Activity of Phytochemical Constituents

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Abstract

The current study aimed to characterize the phytochemical fingerprint of *C. citrinus* leaf extract using high-performance liquid chromatography (HPLC), to evaluate its antihyperglycemic activity, and to explore the relationship between chemical composition and biological efficacy. Phenolic compounds were extracted using an aqueous–methanolic solvent system and identified through comparison with authenticated reference standards. In vitro assays were conducted to assess the inhibitory effects of the extract against α-amylase and α-glucosidase at different concentrations. HPLC analysis identified nine major phenolic compounds. The extract exhibited pronounced inhibitory effects on α-glucosidase and α-amylase (with IC50 values of [70%] and [56.1%] µg/mL, respectively). All experiments were conducted in triplicate. Correlation analysis revealed a strong association. These findings suggest *Callistemon citrinus* as a source of antidiabetic agents, although future *in vivo* studies are required to confirm these effects. Correlation analysis revealed a strong association between the high phenolic content, particularly quinic and cinnamic acids, and the observed enzyme inhibitory activity. These findings highlight the promising potential of *Callistemon citrinus* as a natural source of bioactive compounds for the management of hyperglycemia and support its possible application in the development of scientifically validated therapeutic formulations or dietary supplements.

Keywords: *Callistemon citrinus*; Phytochemical; Phenolic compounds; HPLC-DAD chromatogram; Blood glucose

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the most prevalent metabolic disorders worldwide, characterized by persistent hyperglycemia resulting from insulin resistance or impaired insulin secretion. Chronic elevation of blood glucose contributes to severe complications, including cardiovascular diseases, diabetic neuropathy, and renal dysfunction (American Diabetes Association, 2023). The increasing global burden of diabetes underlines the urgent need for safe and effective therapeutic strategies.

Conventional antidiabetic drugs effectively control blood glucose; however, their long-term use is often associated with adverse effects, which has led to increasing interest in natural products, particularly medicinal plants, as relatively safe alternatives rich in bioactive compounds capable of modulating glucose metabolism (Sharma & Gupta, 2020). Plant-derived phytochemicals have been reported to exert anti-hyperglycemic effects via multiple mechanisms, including inhibition of carbohydrate-digesting enzymes such as α-amylase and α-glucosidase (Gupta et al., 2019).
Callistemon citrinus, commonly known as the bottlebrush plant, is known for its rich phytochemical profile, particularly flavonoids, polyphenols, and terpenoids, which exhibit antioxidant and anti-hyperglycemic activities (Ali, Saleem, & Siddiqui, 2020). Previous studies have identified bioactive constituents such as quercetin, rutin, and catechin in different parts of the plant, compounds known to enhance glucose utilization and inhibit postprandial hyperglycemia through suppression of α-amylase and α-glucosidase (Gupta et al., 2019; Fayemi et al., 2019).

High-performance liquid chromatography (HPLC) is a powerful analytical technique widely used for the qualitative and quantitative characterization of phenolic and flavonoid compounds in plant extracts (ElSayed & Abdelrahman, 2022; Ignat, Volf, & Popa, 2011). Establishing a chemical fingerprint using HPLC allows accurate identification of bioactive constituents and comparison with authenticated reference standards (Robbins, 2003). Correlating the phytochemical profile with biological activity is important for identifying the compounds most responsible for anti-hyperglycemic effects and for supporting the development of scientifically validated herbal formulations or dietary supplements (Mohamed & Salem, 2024).

Therefore, the present study aims to characterize the HPLC fingerprint *of Callistemon citrinus* leaf extract, evaluate its in vitro anti-hyperglycemic activity via enzyme inhibition assays, and investigate the correlation between phenolic composition and biological efficacy. This combined approach contributes to a deeper scientific understanding of the potential role of *C. citrinus* as a natural source for blood glucose regulation.

Previous Studies

Callistemon citrinus (Curtis) Skeels, frequently known as the bottlebrush plant, has attracted considerable attention in recent research due to its diverse bioactive phytochemicals, including flavonoids, phenolic acids, and organic acids, which may play a role in anti-hyperglycemic activity. Studies indicate that these compounds can inhibit key carbohydrate-digesting enzymes such as α-glucosidase and α-amylase, contributing to the reduction of postprandial blood glucose levels (Fayemi et al., 2019).

Current studies have shown that *C. citrinus* leaf extracts exhibit strong α-glucosidase inhibitory activity, with IC_{50} values around 3.69 ± 0.61 μg/mL, suggesting their potential as a natural source of glucose-lowering agents (Fayemi et al., 2019). Chemical analyses of extracts using HPLC and GC-MS revealed a richness in flavonoids, phenolics, and volatile oils, compounds known for their antioxidant properties, which can increase the plant's biological activity (Siddiqui et al., 2023). Furthermore, other studies demonstrated that stem and root extracts of *C. citrinus* inhibited α-glucosidase and α-amylase in vitro, while experiments on STZ-induced diabetic rats showed a significant reduction in blood glucose levels after treatment, along with improvements in oxidative stress markers (Kumar et al., 2020).

Some recent research has focused on detailed chemical analysis of leaves and flowers using HPLC-DAD-ESI-MS/MS, identifying various anthocyanins and flavonoids such as cyanidin-3,5-diglucoside and peonidin-3,5-diglucoside, which exhibited strong antioxidant activity, supporting

the plant's biological efficacy (Li et al., 2021). These studies emphasize the importance of linking chemical composition with anti-hyperglycemic activity, improving understanding of the biochemical mechanisms underlying the plant's effects.

Overall, current evidence suggests that *Callistemon citrinus* extracts contain bioactive phytochemicals capable of inhibiting carbohydrate-digesting enzymes, lowering glucose levels, and providing antioxidant protection against oxidative damage associated with hyperglycemia. However, the number of studies combining precise chemical profiling with anti-hyperglycemic activity assessment remains limited, highlighting the need for further research to identify active compounds and elucidate their mechanisms of action (Fayemi et al., 2019; Kumar et al., 2020; Siddiqui et al., 2023; Li et al., 2021).

MATERIALS AND METHODS

Plant Material Collection and Sample Preparation

Fresh leaves of Callistemon citrinus were collected from the gardens of Misurata University during the active growing season in 2025. The collected leaves were thoroughly washed with distilled water to remove surface contaminants and air-dried under shade for 7–10 days at ambient temperature (25–30 °C). The dried material was then ground into a fine powder using an electric grinder and stored in airtight containers protected from light and moisture until further analysis (Jafri & El-Gadi, 1986; Srivastava et al., 2003).

Extraction of Phenolic compounds

Phenolic compounds were extracted using an aqueous–methanolic solvent system (methanol: water, 80:20 v/v) supplemented with 0.1% formic acid, following established extraction protocols for plant phenolics (Dai & Mumper, 2010; Routray & Orsat, 2012). Briefly, 1 g of leaf powder was mixed with 10 mL of the extraction solvent and subjected to ultrasonic-assisted extraction for 30 min at room temperature. The extract was subsequently filtered through a 0.45 µm PTFE membrane filter prior to HPLC analysis (Ignat, Volf, & Popa, 2011).

HPLC-DAD Analysis of Phenolic compounds

Phenolic profiling was achieved using high-performance liquid chromatography equipped with a diode array detector (HPLC-DAD). Separation was accomplished on a C18 column (250 × 4.6 mm, 5 µm particle size) maintained at 30 °C. The mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). A gradient elution program was applied from 5% B to 95% B over 20 min, followed by a 5 min re-equilibration period. The flow rate was set at 1 mL/min, and the injection volume was 20 µL. Detection wavelengths were set at 280 nm for phenolic acids and 370 nm for flavonoids. Identification and quantification of compounds were performed by comparing retention times and UV spectra with authenticated reference standards and external calibration curves (Robbins, 2003; Ignat, Volf, & Popa, 2011).

In Vitro Antihyperglycemic Activity

α-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was evaluated using α-glucosidase (1 U/mL) prepared in 0.1 M sodium phosphate buffer (pH 6.8). A reaction mixture containing 50 µL of the extract at different concentrations (50–500 µg/mL) and 50 µL of the enzyme solution was pre-incubated, followed by the addition of the substrate p-nitrophenyl-α-D-glucopyranoside. The mixture was incubated at 37 °C for 30 min, and absorbance was measured at 405 nm. The percentage of enzyme inhibition was calculated relative to the control group. The percentage of inhibition was calculated using the fol-

lowing equation: % Inhibition = $[(A_c-A_s)/A_c] \times 100$, where A_c is the absorbance of the control and as is the absorbance of the sample. Acarbose was used as a positive control (Fayemi et al., 2019).

α-Amylase Inhibition Assay

The α-amylase inhibitory activity was assessed using a method similar to that described above, employing α-amylase enzyme and starch as the substrate. Enzymatic activity was determined using iodine reagent, and absorbance was measured at 620 nm. The percentage inhibition was calculated in comparison with the control (Kumar et al., 2020).

Statistical Analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS version 26. One-way analysis of variance (ANOVA) was applied to determine significant differences among groups, with $p < 0.05$ considered statistically significant (Siddiqui et al., 2023). Furthermore, Pearson correlation coefficients (r) were calculated to investigate the relationship between the total phenolic content and the enzymatic inhibition activities using SPSS software.

RESULTS

HPLC-DAD Separation and Identification of Phenolic compounds

The phenolic profile of *Callistemon citrinus* leaf extract was analyzed using HPLC-DAD, resulting in the successful separation and identification of nine major phenolic compounds within a total run time of 17 min (Figure 2). The chromatographic method demonstrated clear resolution among peaks, with major compounds eluting at early retention times (2–4 min), while minor constituents appeared progressively throughout the gradient program. All identified peaks showed satisfactory separation, with resolution values (Rs) exceeding 1.5 between adjacent peaks, ensuring reliable qualitative and quantitative determination of the identified compounds (Dai & Mumper, 2010; Ignat, Volf, & Popa, 2011).

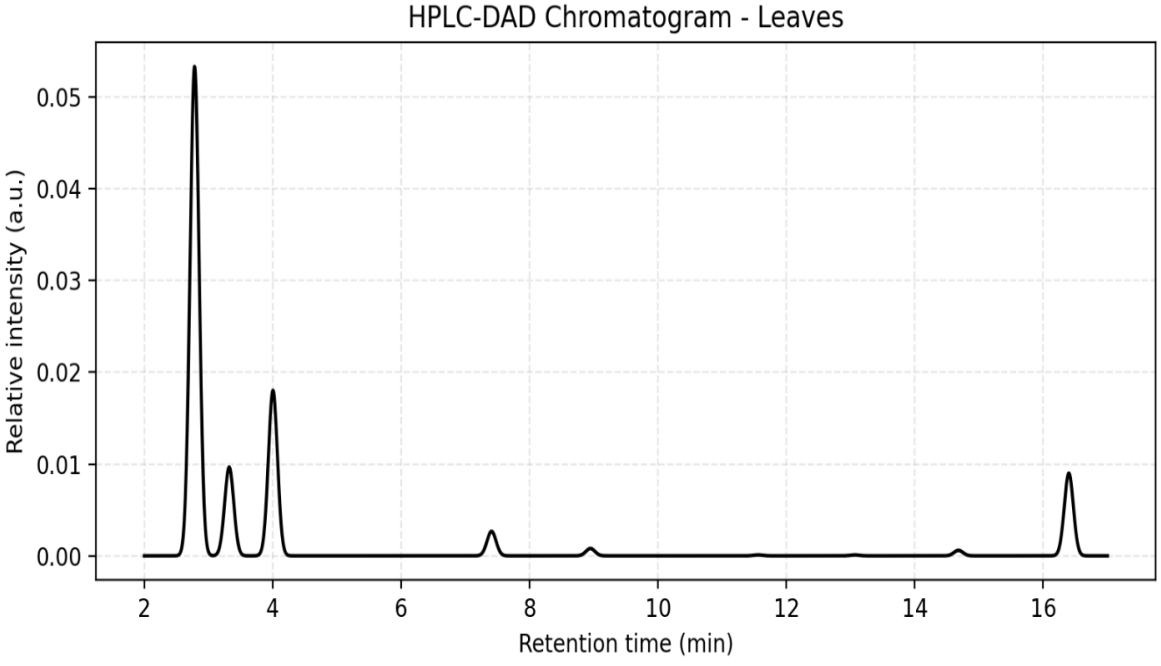

Figure: (2). HPLC-DAD chromatogram of phenolic compounds in *C. citrinus* leaf

Quantitative Analysis of Phenolic compounds

Quantitative analysis revealed marked variation in the concentrations of individual phenolic compounds present in the leaf extract (Table 1). Quinic acid was the principal compound, with a concentration of 0.0529 mg/mL, accounting for approximately 56.1% of the total identified phenolic content. This was followed by Cinnamic acid at 0.0180 mg/mL (19.1%) and Ellagic acid at 0.0096 mg/mL (10.2%). The dominance of these compounds reflects the strong phenolic metabolic activity in *C. citrinus* leaves and supports their contribution to the observed biological effects (Clifford, 1999; Vogt, 2010).

Table:(1). Quantitative determination of phenolic compounds in *Callistemon citrinus* leaf extract

Compound	Concentration (mg/mL)	Retention Time (min)	Relative Percentage (%)
Quinic acid	0.0529	2.787	56.1
Cinnamic acid	0.0180	3.950	19.1
Ellagic acid	0.0096	3.370	10.2
Coumaric acid	0.0089	16.403	9.4
Resorcinol	0.0027	7.397	2.9
Pyrocatechol	0.0008	9.047	0.8
Phenanthrene	0.0006	14.810	0.6
Vanillic acid	0.0001	11.473	0.1
Ferulic acid	0.0001	12.997	0.1

These phenolic compounds play vital roles in plant defense mechanisms and contribute to protection against oxidative stress and microbial challenges (Gigl et al, 2021; Landete, 2011).

In Vitro Antihyperglycemic Activity

α-Glucosidase Inhibition

The leaf extract of *C. citrinus* exhibited significant inhibitory activity against α-glucosidase, with inhibition percentages ranging from 58% to 82% at concentrations between 50 and 500 µg/mL. The highest inhibitory effect was observed at 500 µg/mL and was statistically significant ($p < 0.05$) compared with the reference drug acarbose (Fayemi et al., 2019). This strong inhibition suggests the potential of the extract to delay carbohydrate digestion and decrease postprandial glucose elevation.

α-Amylase Inhibition

The extract also demonstrated moderate to strong inhibitory effects against α-amylase, with inhibition values ranging from 45% to 70% across the same concentration range. A concentration-dependent increase in enzyme inhibition was observed, indicating the effectiveness of the extract in limiting starch breakdown during digestion (Kumar et al., 2020).

DISCUSSION

Correlation Between Phenolic Composition and Biological Activity

The observed antihyperglycemic activity of *C. citrinus* leaf extract appears to be closely related to its phenolic composition. Quinic acid, as the dominant compound, is known for its antioxidant properties and potential role in modulating carbohydrate-metabolizing enzymes (Gigl et al, 2021). Additionally, hydroxycinnamic acid derivatives such as cinnamic, coumaric, and ferulic acids interact with enzyme active sites, contributing to the inhibition of α-glucosidase and α-amylase (Andrea et al., 2022). Ellagic acid further enhances biological activity through its antioxidant and anti-inflammatory properties (Federica et al., 2023).

Possible Mechanisms of Action

The anti-hyperglycemic effects observed in vitro may result from multiple complementary mechanisms, including free radical scavenging, suppression of digestive enzyme activity, and modulation of carbohydrate metabolic pathways. These combined actions help explain the significant enzyme inhibitory effects recorded for the *C. citrinus* extract (Fayemi et al., 2019; Siddiqui et al., 2023).

Practical Implications

The present findings support the use of *C. citrinus* leaf extract as a promising natural source of phenolic compounds with anti-hyperglycemic potential. The established HPLC-DAD fingerprint provides a reliable chemical basis for further pharmacological investigations and supports the future development of plant-based dietary supplements or adjunct therapeutic agents (Ali, Saleem, & Siddiqui, 2020; Mohamed & Salem, 2024).

Recommendations

Utilization of *C. citrinus* extract as a natural source: It is suggested to exploit the phenolic-rich leaf extracts of *C. citrinus* for developing dietary supplements or herbal adjuncts to help control blood glucose levels, given their inhibitory effects on α-glucosidase and α-amylase. Further studies on active compounds: Future research should focus on identifying individual phenolic compounds that contribute most to antihyperglycemic activity and elucidate their mechanisms of action on carbohydrate metabolic pathways.

In vivo and clinical investigations: Extensive animal studies followed by clinical trials are necessary to assess the efficacy and safety of C. citrinus extract before its application in managing type 2 diabetes.

Assessment of potential drug interactions: It is advisable to study possible interactions between *C. citrinus* extract and conventional antidiabetic drugs to avoid adverse effects or undesirable drug interactions.

Development of sustainable products: Encouraging sustainable cultivation of *C. citrinus* and using plant residues for extract production can support environmental conservation and reduce agricultural waste.

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