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# RP-HPLC AND HPTLC-BASED PHYTOCHEMICAL STUDIES OF ENDEMIC *NEPETA CADMEA* BOISS. AND ITS EFFECTS ON CARBOHYDRATE DIGESTIVE ENZYMES

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**ABSTRACT.** This study investigated the *in vitro* inhibitory potential of the methanol extract of the aerial parts of Nepeta cadmea on *α*-glucosidase and *α*-amylase, which are known as key enzymes related to type 2 diabetes mellitus. To determine the active constituents of the endemic Lamiaceae plant, validated RP-HPLC and HPTLC techniques were used. The methanol extract demonstrated moderate and dose-dependent inhibitory activity, with inhibition rates of 43.57 ± 4.32% against *α*-glucosidase at 2 mg/mL and 36.74 ± 7.23% against *α*-amylase at 1 mg/mL. Phytochemical analysis revealed that rosmarinic acid (0.6290 ± 0.0095 g/100g dw) was the predominant phenolic compound, followed by chlorogenic acid (0.0429 ± 0.0012 g/100g dw) and caffeic acid (0.0027 ± 0.0002 g/100g dw). These findings suggest that this endemic Lamiaceae plant may have potential as a natural therapeutic agent for managing diabetes mellitus. Further studies are warranted to explore its mechanisms of action and to evaluate its efficacy in clinical settings.

***Keywords:*** *α-amylase, α-glucosidase, RP-HPLC, HPTLC, Nepeta cadmea*

## INTRODUCTION

The genus *Nepeta* L. (Lamiaceae) is known to comprise approximately 250 species worldwide. Reports indicate the distribution of *Nepeta* species across Southwest and Central Asia, North America, North Africa, Europe,

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and the Mediterranean regions. In Türkiye, this genus contains a total of 39 species and 50 taxa, 19 of which are endemic [1, 2]. As with most members of the Lamiaceae family, many *Nepeta* species have been traditionally used for colds, stomach aches, and as stimulants. Some *Nepeta* species are also traditionally used as diuretics, sudorific agents, cough suppressants, antispasmodic agents, asthma treatments, antipyretics, and sedatives; for nervous disorders and depression; as spices; and in herbal teas [2, 3]. Numerous studies on *Nepeta* species have revealed that these plants contain terpenoid compounds such as monoterpenes, sesquiterpenes, diterpenes, triterpenes, and phenolic compounds in addition to nepetalactones and their glycosidic forms. Due to their pleasant aroma and generally high monoterpene content, these plants yield highly valued essential oils. Terpenoids and flavonoids are the dominant components in the chemical composition of the *Nepeta* genus [4, 5]. *Nepeta cadmea* Boiss., a species of the *Nepeta* genus, is known in Turkish as “honaz pisikotu” and is endemic to Türkiye. *N. cadmea* is a perennial herbaceous species rich in essential oils [2, 6, 7]. Phytochemicals found in *N. cadmea* include 4a*α*,7α,7a*α*-nepetalactone; 1,5,9-epideoxyloganic acid; (*E*)-rosmarinic acid; eugenyl *β*-*D*-glucopyranoside; eugenyl-*O*-*β*-apiofuranosyl-(1→6)-*O*-*β*-glucopyranoside; lariciresinol 4'-*O*-*β*-*D*-glucopyranoside; guloside (corcoyonoside C); icariside B2 and icariside B2 [8, 9].

Diabetes mellitus (DM) is a common metabolic disease worldwide, and type 2 diabetes (T2DM), in particular, is characterized by insulin resistance and persistently high blood glucose levels. In patients with T2DM, postprandial hyperglycemia is associated with impaired starch digestion, a process in which the enzymes *α*-glucosidase and *α*-amylase play key roles. Inhibition of these enzymes is being investigated as an effective therapeutic strategy to regulate blood sugar levels and manage diabetes [10]. Among *Nepeta* species, a 70% ethanol extract of *N. cataria* L. was found to have a moderate inhibitory effect on enzymes responsible for carbohydrate hydrolysis. The literature has reported that the methanol extract of *N. hindostana* (B. Heyne ex Roth) Haines exhibits a significant antidiabetic effect in a streptozotocin-induced diabetes model [11-13].

The aim of this study was to perform qualitative and quantitative analyses of the methanol extract obtained from the aerial parts of *N. cadmea* for phenolic compounds. For this purpose, a Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed to determine the amounts of the main compounds in the plant extract. Furthermore, High-Performance Thin-Layer Chromatography (HPTLC) analysis was performed to identify the active ingredient groups contained in the methanol extract of the plant, thus strengthening the specificity of the study. Another important objective of this study was to test the *in vitro* antidiabetic potential of *N. cadmea*. In

this context, the potential antidiabetic effects of the plant were evaluated by testing its *α*-glucosidase and *α*-amylase inhibitory activities, which play an effective role in regulating blood glucose levels and managing diabetes.

## RESULTS AND DISCUSSION

Studies on *Nepeta* species have focused primarily on their essential oil components. In this study, which investigated the antidiabetic potential of an extract prepared with methanol from the aerial parts of *N. cadmea* collected from the Denizli region, *α*-glucosidase and *α*-amylase enzymes were used. Furthermore, qualitative and quantitative analyses of the phenolic compounds in the extract were performed using RP-HPLC, and TLC screening of phenolic compounds was conducted, and the HPTLC technique was applied for the first time for this species.

As a result of these studies, the highest inhibition of the *α*-glucosidase enzyme by *N. cadmea* methanol extract was 43.57 ± 4.32% at a concentration of 2 mg/ml. The inhibition value of the reference compound, acarbose, at the same concentration was 100%. The highest inhibition of the *α*-amylase enzyme by *N. cadmea* methanol extract was 36.74 ± 7.23% at a concentration of 1 mg/ml (Table 1). Accordingly, *N. cadmea* methanol extract can be considered a moderate *α*-glucosidase and *α*-amylase inhibitor. In their 2019 study, Sarikurkcu and colleagues (2019) investigated the *α*-glucosidase and *α*-amylase inhibitory activity of extracts prepared using methanol via Soxhlet extraction from the aerial parts of *N. cadmea*. The study concluded that the inhibitory activity of *N. cadmea* on *α*-glucosidase and *α*-amylase was found to be 2.02 and 0.24 mmol ACEs/g extract, respectively ($p<0.01$). These results demonstrated that the plant extract has a weak inhibitory effect on both enzymes [5]. Considering the differences between our research and the 2019 study by Sarıkurkcu et al., to the best of our knowledge this study is the first HPTLC-based phenolic profile identification for *N. cadmea*, and rosmarinic acid is adressed as the major compound in the methanol extract. Also, the determination of moderate and dose-dependent inhibitory activity of *α*-glucosidase is important for the originality of the study.

In another study conducted by Sarikurkcu et al. (2018) the *α*-glucosidase and *α*-amylase inhibitory activities of the essential oil obtained from the aerial parts of *N. cadmea* were investigated. The study results showed that the inhibitory activity of *N. cadmea* essential oil on α-glucosidase ($IC_{50}$: 5.93 ± 0.18 mg/mL) was very low compared to acarbose ($IC_{50}$: 0.99 ± 0.01 mg/mL), while its inhibitory value on *α*-amylase ($IC_{50}$: 1.35 ± 0.02 mg/mL) was very similar to that of acarbose ($IC_{50}$: 1.33 ± 0.01 mg/mL). The findings indicated that the essential oil was highly effective on *α*-amylase [14].

**Table 1.** Inhibitory activity of *N. cadmea* methanol extract on *α*-glucosidase and *α*-amylase enzymes

| Samples | Concentration (mg/ml) | Inhibition % ± S.D. | |
|---|---|---|---|
| | | ***α*-Glucosidase** | ***α*-Amylase** |
| *N. cadmea* extract | 0.5 | n.a. | n.a. |
| *N. cadmea* extract | 1 | 17.14 ± 2.18*** | 36.74 ± 7.23*** |
| *N. cadmea* extract | 2 | 43.57 ± 4.32*** | n.a. |
| Acarbose | 0.5 | 100 ± 0.00*** | n.a. |
| Acarbose | 1 | 99.86 ± 0.24*** | 78.88 ± 8.53*** |
| Acarbose | 2 | 100 ± 0.00*** | 100 ± 0.00*** |

-: No activity (n.a.), S.D.: Standard Deviation, ***p<0.001
(compared with acarbose values at the same concentration)

In this study, in addition to RP-HPLC assay, TLC, and HPTLC techniques were also used for the phytochemical analysis of *N. cadmea*. Based on the available literature, HPTLC analysis was performed for the first time on this species (Figures 1 and 2). The phenolic profile of the *N. cadmea* methanol extract was determined by RP-HPLC targeting the following compounds: gallic acid, chlorogenic acid, caffeic acid, sinapic acid, ellagic acid, protocatechuic acid, *p*-coumaric acid, ferulic acid, rosmarinic acid, rutin, luteolin-7-glucoside, quercetin, luteolin, apigenin, and kaempferol (Figures 3 and 4). In addition, quantitative analyses were carried out specifically for rosmarinic acid, chlorogenic acid, and caffeic acid. The validation parameters of the quantitatively analyzed compounds are presented in Table 2. The study revealed that the plant contained significant amounts of rosmarinic acid, chlorogenic acid, and caffeic acid. According to the quantitative determination results, the rosmarinic acid, chlorogenic acid, and caffeic acid rates in the plant were determined as 0.6290 ± 0.0095 g/100g dw, 0.0429 ± 0.0012 g/100g dw, and 0.0027 ± 0.0002 g/100g dw, respectively (Table 3).

Kaska and colleagues (2018) carried out phytochemical analysis of ethanol extract prepared from aerial parts of *N. cadmea* using RP-HPLC. The study reported that the ethanol extract was rich in chlorogenic acid (1050.74 µg/g) and caffeic acid (3683.34 µg/g) [7]. In another study where the phytochemical content of *N. cadmea* was evaluated using the RP-HPLC technique, it was found that the amounts of chlorogenic acid (3.30 ± 0.12 mg/g) and caffeic acid (0.47 ± 0.03 mg/g) were high, but the plant did not contain rosmarinic acid [5]. RP-HPLC analysis indicated that methanol extracts of flowers, leaves, and roots of *N. humilis*, another *Nepeta* species, contained only rosmarinic acid. It has been reported that the flower extract

has the highest rosmarinic acid content (0.397 ± 0.01 g/100 g dry weight) [15]. These results indicated that the levels of chlorogenic acid and caffeic acid detected in our study were lower, while rosmarinic acid levels were higher, compared to previous studies. These differences may be attributed to various environmental and methodological factors, such as the extraction technique used, the type of solvent, the geographical region where the plant was collected, the time of collection, and seasonal factors. Our RP-HPLC analysis identified rosmarinic acid, previously reported by Takeda et al. (1998) [9], to be the main compound isolated from *N. cadmea*. Furthermore, a study by Ngo and Chua (2018) [16], revealed that rosmarinic acid exhibited high inhibitory activity, particularly against the *α*-glucosidase enzyme, making it a promising natural candidate for the treatment of type 2 diabetes. In the literature, chlorogenic acid, caffeic acid, and rosmarinic acid compounds found in *N. cadmea* have been reported to have inhibitory effects on *α*-glucosidase and *α*-amylase enzymes [17-20]. In our study, the methanol extract of the aerial parts of *N. cadmea* had moderate *α*-glucosidase and *α*-amylase inhibitory activity, suggesting that these phenolic compounds may exhibit inhibitory effects alone or through a synergistic interaction. In particular, the potential of rosmarinic acid, both alone and in combination with other phenolics, has led to the idea that this molecule should be considered an important phytochemical candidate for antidiabetic activity.

**Table 2.** $r^2$, LOD, LOQ values, mean area, SD, and % RSD data for chlorogenic acid, caffeic acid, and rosmarinic acid

| Compound | Standard curve | $r^2$ | LOD (mg/L) | LOQ (mg/L) | Mean area, n= 9 | SD | %RSD |
|---|---|---|---|---|---|---|---|
| Chlorogenic acid | y= 22083x – 6.4807 | 0.9999 | 0.112 | 0.370 | 7.35 | 0.1309 | 1.78 |
| Caffeic acid | y= 56891x + 30.81 | 0.9996 | 0.021 | 0.069 | 5.13 | 0.0997 | 1.94 |
| Rosmarinic acid | y= 28820x – 26.234 | 0.9999 | 0.073 | 0.240 | 130.79 | 0.1572 | 1.95 |

**Table 3.** The content of phenolic compounds in *N. cadmea* aerial parts (g/100 g dry weight of the plant)

| Compound | *N. cadmea* |
|---|---|
| Chlorogenic acid | 0.0429 ± 0.0012 |
| Caffeic acid | 0.0027 ± 0.0002 |
| Rosmarinic acid | 0.6290 ± 0.0095 |

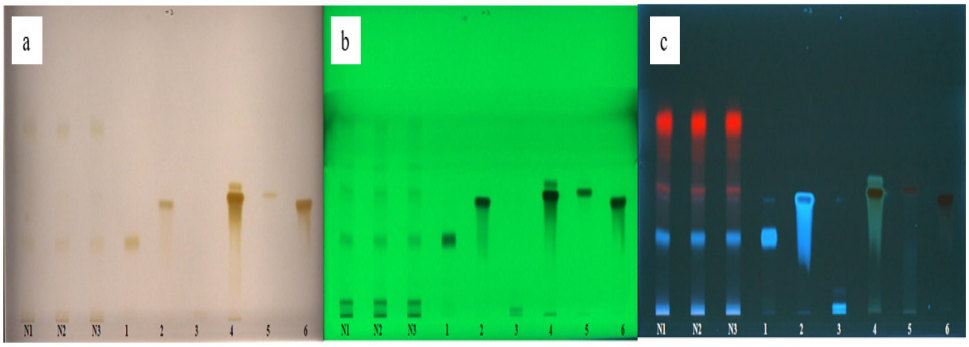


**Figure 1**. HPTLC chromatograms of the methanol extract of the aerial parts of *N. cadmea* and standard compounds in the solvent system Toluene: Ethyl Acetate: Formic Acid (5:4:1), visualized under a) daylight, b) UV 254 nm, c) UV 366 nm (N1, N2, N3: Extract; 1: Rosmarinic Acid, 2: Caffeic Acid, 3: Chlorogenic Acid, 4: Quercetin, 5: Apigenin, 6: Luteolin)

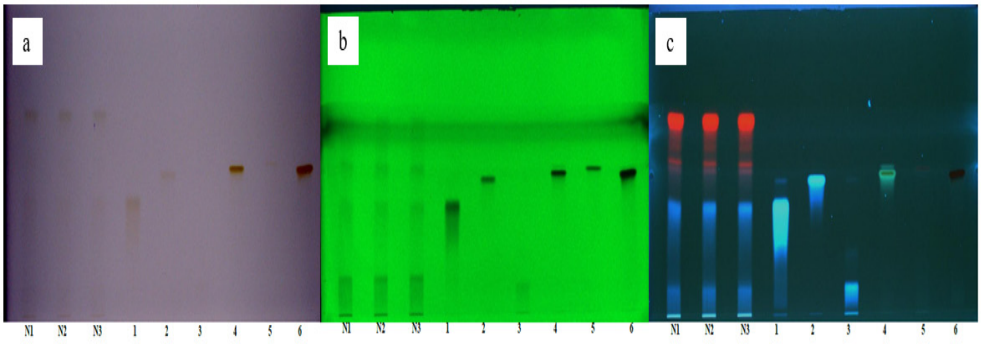


**Figure 2**. HPTLC chromatograms of the methanol extract of the aerial parts of *N. cadmea* and standard compounds in the solvent system Chloroform: Ethyl Acetate: Acetone: Formic Acid (4:3:2:1), visualized under a) daylight, b) UV 254 nm, and c) UV 366 nm (N1, N2, N3: Extract; 1: Rosmarinic Acid, 2: Caffeic Acid, 3: Chlorogenic Acid, 4: Quercetin, 5: Apigenin, 6: Luteolin)

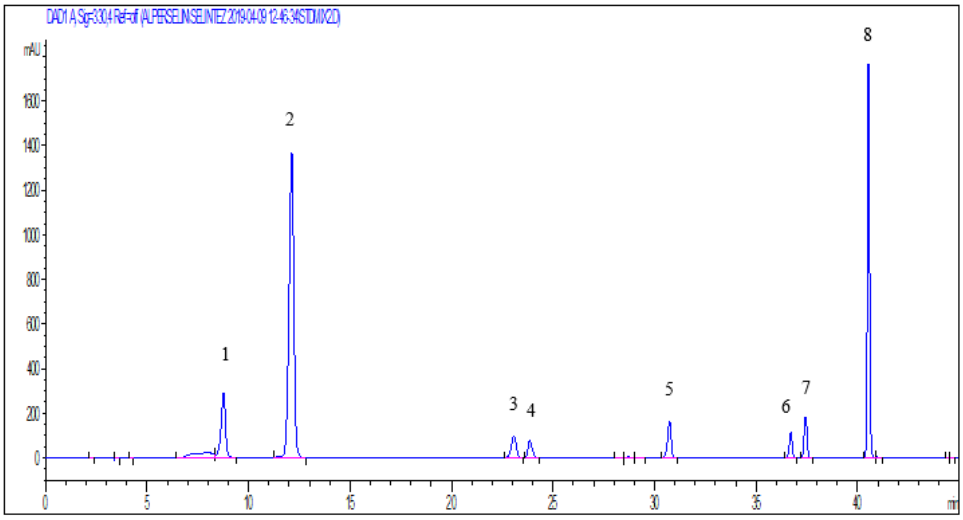


**Figure 3**. RP-HPLC chromatogram of some standard compounds at 330 nm (in order: chlorogenic acid (1), caffeic acid (2), rutin (3), ellagic acid (4), rosmarinic acid (5), quercetin (6), luteolin (7), apigenin (8)

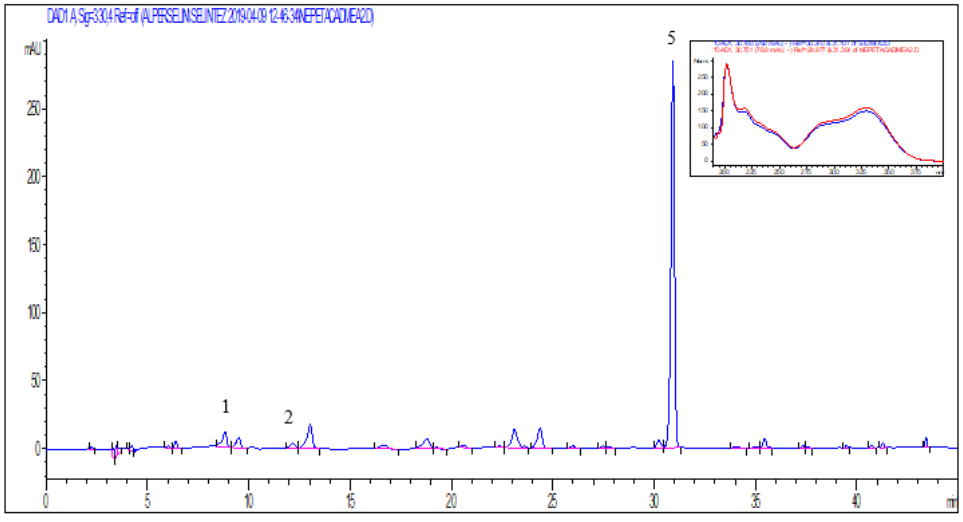


**Figure 4**. RP-HPLC chromatogram of the methanol extract of the aerial parts of *N. cadmea* at 330 nm, chlorogenic acid (1), caffeic acid (2), rosmarinic acid (5) and overlaid UV spectra of standard rosmarinic acid and the rosmarinic acid detected in the extract

## CONCLUSIONS

In this study, the *in vitro* antidiabetic potential of a methanol extract of *N. cadmea* aerial parts was determined to have a moderate inhibitory effect, compared to acarbose, on the enzyme *α*-glucosidase, which plays a role in carbohydrate digestion. Both RP-HPLC and HPTLC analyses provided detailed characterization of the plant's phenolic compound content, revealing rosmarinic acid as the main component, followed by chlorogenic and caffeic acids. The higher inhibitory effect observed in this study, compared to previous studies, may be due to the synergistic effect of the phenolic compounds present in the extract, especially rosmarinic acid. This study represents the first application of HPTLC to *N. cadmea*, introducing an innovative analytical approach that enhances the accuracy and reproducibility of phytochemical profiling. In conclusion, this study provided a more detailed characterization of the phytochemical structure of *N. cadmea* and showed that the plant can be considered a natural resource that could lead to different *in vitro* and *in vivo* studies for the management of postprandial hyperglycemia in type 2 diabetes mellitus.

## EXPERIMENTAL SECTION

### *Plant material*

*N. cadmea* was collected in its flowering stage from Denizli Kızılcabölük, Turkey. Voucher specimen has been deposited in the Herbarium of Ankara University Faculty of Pharmacy under the herbarium code of AEF 28879. The plant was identified by Prof. Dr. Mehmet Çiçek from Pamukkale University and Assoc. Prof. Dr. Gülderen Yılmaz from Ankara University.

### *Preparation of extract and standards*

Dried and milled aerial parts of the plant were extracted twice with methanol using a magnetic stirrer for 4 h (50 °C, 600 rpm). The filtered extracts were combined, and methanol (MeOH) was completely evaporated by a rotary evaporator (Buchi-R200). The yield of the extract was 14.235%. Phenolic compounds were purchased from Sigma (Germany): apigenin (A3145), caffeic acid (C0625), chlorogenic acid (C3878), ellagic acid (E2250), ferulic acid (128708), gallic acid (G7384), kaempferol (K0133), luteolin (L9283), luteolin-7-glucoside (49968), p-coumaric acid (C9008), protocatechuic acid (03930590), quercetin (Q4951), rosmarinic acid (536954), rutin (R5143), and sinapic acid (D7927).

### *Assay for α-amylase inhibitory activity*

The *α*-amylase inhibitory activity of the methanol extract of *N. cadmea* was determined by the method of Ali et al. (2006) [21]. Porcine pancreatic *α*-amylase type VI (EC 3.2.1.1, Sigma) was dissolved in distilled water. As substrate solution, potato starch (0.5%, w/v) in phosphate buffer (pH 6.9) was used. Experiments were carried out with three replicates. The reaction was initiated by the addition of the enzyme solution. Then the tubes were incubated at 37 °C for 3 min. After the addition of substrate, the tubes were incubated at 37 °C for 5 min. Then, DNS (3,5-dinitrosalicylic acid) colour reagent solution was added to the mixture and put into an 85 °C heater. After 15 min, distilled water was added to the tubes, and the tubes were cooled. Absorbances of the mixtures were read at 540 nm. Acarbose was used as the positive control. Maltose formed as a result of starch hydrolysis was calculated using the maltose calibration equation, and then the inhibition percentage was determined [21].

### *Assay for α-glucosidase inhibitory activity*

*α*-Glucosidase type IV enzyme (Sigma Co., St. Louis, USA) was dissolved in phosphate buffer (0.5 M, pH 6.5). The enzyme solution, extracts, and pure compounds were preincubated in a 96-well microtiter plate for 15 min at 37 °C. After that, the substrate solution [*p*- nitrophenyl-*α*-D-glucopyranoside (NPG), Sigma] was added. The mixture was incubated for 35 min at 37 °C. The increase in the absorption at 405 nm due to the hydrolysis of NPG by *α*-glucosidase was measured by VersaMax ELISA microtiter plate reader [22]. Acarbose (Bayer Group, Turkey), a potent *α*-glucosidase inhibitor, was used as a positive control. The inhibition percentage (%) was calculated by the equation: Inhibition % = $[(A_{Control} - A_{Sample}) / A_{Control}] \times 100$.

### *Phytochemical Analysis*

*TLC and HPTLC analysis*

Prior to HPTLC experiments, TLC trials were performed using various solvent systems to find the most efficient method for the optimum separation of the phenolic compounds: Toluene-ethyl acetate-formic acid (5:4:1, v/v/v), Toluene-ethyl acetate-formic acid (8.5:1.5:0.1, v/v/v), Chloroform-ethyl acetate-acetone-formic acid (4:3:2:1, v/v/v/v), Ethyl acetate-water: formic acid (15:1:1, v/v/v), Ethyl acetate-water-formic acid-acetic acid (100:27:11:11, v/v/v/v), Ethyl acetate-methanol-water (100:16.5:13.5, v/v/v/v). The extracts were applied three times, and standards were applied as rosmarinic acid, caffeic acid, chlorogenic acid, quercetin, apigenin, and luteolin.

Following the determination of the suitable solvent system/s, HPTLC analysis was done using a Camag HPTLC system. Five microliters of the samples were sprayed in the form of 8 mm bands with an Automatic TLC Sampler 4 (ATS 4, CAMAG, Switzerland) onto pre-coated HPTLC glass plates (20 x 10 cm, Si 60 F254, Merck). The distance from the left side and the lower edge was 15 mm and 8 mm, respectively. Linear ascending development was carried out with an Automatic Developing Chamber 2 (ADC 2, CAMAG, Switzerland). Toluene-ethyl acetate-formic acid (5:4:1, v/v/v) and Chloroform-ethyl acetate-acetone-formic acid (4:3:2:1, v/v/v/v) were used as mobile phases. Relative humidity was not controlled. The twin through chamber was saturated for 20 minutes with 25 mL of the mobile phase before development. Ten milliliters of the same mixture were used for development. Plates were air-dried before and after development by ADC2. Documentation of the plates was performed by Reprostar 3 (CAMAG, Switzerland) at 366nm, 254 nm, and daylight. The extracts were applied three times, and the standards were used in the following order: rosmarinic acid, caffeic acid, chlorogenic acid, quercetin, apigenin, and luteolin [23, 24].

*RP-HPLC-DAD analysis*

Chromatographic assay of phenolic compounds was done using a previously validated HPLC-DAD method [25]. An Agilent 1260 Series HPLC system equipped with a quaternary pump, an auto-sampler, a column oven, and a diode-array UV/VIS detector was utilized. Data analysis was performed using Agilent Chemstation. The separation was made on ACE 5 µ C18 (250 × 4.60 mm) column. The mobile phase was a mixture of trifluoroacetic acid 0.1% in water (solution A), trifluoroacetic acid 0.1% in methanol (solution B), and trifluoroacetic acid 0.1% in acetonitrile (solution C). The composition of the gradient was (A:B:C), 80:12:8 at 0 min, 75:15:10 at 8 min, 70:18:12 at 16 min, 65:20:15 at 24 min, 50:35:15 at 32 min, 25:60:15 at 40 min, and 80:12:8 at 45 min. The duration between runs was 2 min. The injection volume was 10 µL for each standard and sample solution. The detection UV wavelength was set at 230, 330, 360, 340, and 280 nm, and the column temperature was set to 30 °C. For the qualitative and quantitative analysis of phenolic compounds by HPLC, some phenolic compounds that are likely to be present in the plant were used as standards. These compounds include gallic acid, chlorogenic acid, caffeic acid, sinapic acid, ellagic acid, protocatechuic acid, *p*-coumaric acid, ferulic acid, rosmarinic acid, rutin, luteolin-7-glucoside, quercetin, luteolin, apigenin, and kaempferol. Quantification was performed by measuring at 330 nm for chlorogenic acid, caffeic acid, and rosmarinic acid using a photo-diode array detector. Quantitative determinations of chlorogenic acid, caffeic acid, and rosmarinic acid were performed, and

the validation parameters such as the coefficient of determination ($r^2$), limits of detection (LOD) and quantification (LOQ), mean area values (x), standard deviation (SD), and percentage relative standard deviation (%RSD) were calculated.

### *Statistical Analysis*

All experiments were performed in triplicate, and data are expressed as the mean ± standard deviation (S.D.). Statistical evaluations, including linear regression, were carried out using Microsoft Excel and GraphPad Instat software. Statistical significance was determined at $p<0.05$ ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

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