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# Identification and Quantification of Phenolic Acids in *Macrotyloma uniflorum* by Reversed Phase-HPLC*

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**Abstract:** Extracts of *Macrotyloma uniflorum* plants were examined as potential sources of phenolic compounds. Reversed phase high performance liquid chromatography (RP-HPLC) with UV detection was employed for the identification and quantification of the phenolic acids. Eight phenolic acids, namely, 3, 4-dihydroxy benzoic, *p*-hydroxy benzoic, vanillic, caffeic, *p*-coumeric, ferulic, syringic and sinapic acids were isolated from an ethanolic extract of *Macrotyloma uniflorum*. The most abundant phenolic acids were *p*-coumaric acid (8.95 mg $10^{-2}$ g of dry sample) and *p*-hydroxy benzoic acid (7.81 mg/100 g of dry sample).

**Key words:** Chromatography, *p*-coumaric acid, ethanol, plant material, isolation, fraction

## INTRODUCTION

Phenolic acids are a large family of secondary metabolites having hydroxyl benzoic or hydroxyl cinnamic structures. They are widely distributed plant constituents. They commonly occur as free acids and their esters, glycosides and bound complexes and they are known to play important roles in plant resistance to pathogens and herbivores, allelopathy, oxidative stress and plant growth regulation. Their role concerns color and sensory characteristics of plants as well as antioxidant properties of plant-based food (Moure *et al*., 2001). This role in the organoleptic properties of foods has been a big interest of analytical and food chemists (Canas *et al*., 2003). Additionally, the content and profile of phenolic acids, their effect on fruit maturation and prevention of enzymatic browning, and their roles as food preservatives have been evaluated too (Robbins, 2003; Nurmi *et al*., 2006). Recent interest comes from their potential protective role against diseases that may be related to oxidative damage, such as coronary heart diseases or cancers (Gomes *et al*., 2003; Nichenametla *et al*., 2006).

Dietary polyphenols such as phenolic acids are considered to be powerful antioxidants. Their antioxidant activity is much higher *in vitro* than of well-known vitamin antioxidants (Tsao and Deng, 2004). Antioxidation is, however, only one of the many mechanisms through which polyphenols can exert their actions. Polyphenols have been reported demonstrate antimicrobial (Taguri *et al*., 2006; Rauha *et al*., 2000), antiviral (Perez, 2003), antimutagenic (Lairon and Amiot, 1999), anticarcinogenic (Aaby *et al*., 2004), anti-inflammatory (Dos Santos *et al*., 2006; Parr and Bolwell, 2000), antiproliferative and vasodilatory actions (Lule and Xia, 2005).

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The recent methods dealing with the analysis of phenolics are summarized in review articles (Naczk and Shahidi, 2004; Molnár-perl and Fuzfai, 2005; Rijke *et al*., 2006). It can be seen that the high performance liquid chromatography (HPLC) occupies a leading position in the analysis of phenolics. In general, HPLC separations are based on $C_{18}$ reversed-phased columns and a binary solvent gradient. The mobile phase usually consists of an aqueous solution of acid and an organic solvent (acetonitrile or methanol).

*Macrotyloma uniflorum* Linn (Bengali name-Kurti kalai, English name-horse gram, Family- Fabaceae) is a herbaceous plant with annual branches, suberect or twining, leaflets 2.5-5 cm and widely distributed throughout Bangladesh but abundant in Rajshahi and Dinajpur districts (Kirtikar and Basu, 1998). It is famous for its medicinal uses because different parts of the plants are used for the treatment of heart conditions, asthma, bronchitis, leucoderma, urinary discharges and for treatment of kidney stones (Ghani, 2003). Indeed, *Macrotyloma uniflorum* could play a role in antioxidation (Reddy *et al*., 2005) as when this plants were exposed to toxic levels of lead, several enzymes showed a pivotal role against oxidative injury. *Macrotyloma uniflorum* has the greatest potential for further utilization as nutraceuticals, forage and food for malnourished and drought-prone areas of the world (Morris, 2008). Herbal medicine is part and parcel of the much needed health care in most of the developing countries including Bangladesh. A part of our investigations on the medicinal plants, we investigated *M. uniflorum* and isolated Kaempferol-3-*O*-β-D-glucoside, β-sitosterol and stigmasterol (Kawsar *et al*., 2003) and we very recently reported the cytotoxicity assessment (Kawsar *et al*., 2008a) and antimicrobial activities (Kawsar *et al*., 2008b) of this plant. The aim of this study was further conducted to identification and quantification of phenolic acids detail in *M. uniflorum* plant by using RP-HPLC.

## MATERIALS AND METHODS

### Plant Material

*Macrotyloma uniflorum* (Fabaceae) was collected from the village, Susunda of Muradnagar, Comilla, Bangladesh in March 2002. The botanical identification and voucher specimen was deposited at the Bangladesh National Herbarium (BNH) (DACB Accession No. 28264). The whole plants were cleaned, air-dried and followed by drying in an oven at 40°C. The dried plants were powdered by grinding in a cyclotec-grinding machine (200 mesh). The powdered plant (100 g) was extracted as shown in Fig. 1.

### Extractions

The plant powdered (100 g) was treated with ethanol (99%, 3×500 mL, 8 h, Fig. 1) at room temperature (25°C). The ethanol extract (A) was collected by suction through a Buchner funnel. The residue was air dried for 8 h followed by drying at 40°C for 18 h in an oven and ground to a powder. This was further extracted with aqueous 80% ethanol (2×500 mL, 30 min) at reflux temperature (B). The combined ethanol extract (A+B) was evaporated to remove ethanol. The final volume was adjusted to 500 mL by adding water and was distributed between chloroform to remove lipids and water (3×500 mL).

### Isolation of Phenolic Acids

The water fraction was adjusted to pH 2.5 by adding 1 M HCl. It was then extracted with ethyl acetate (3×200 mL) and the combined extract was evaporated after drying with anhydrous sodium sulphate (EA-1). The residual aqueous solution was divided into two portions. To one portion sodium hydroxide solution (2 M) was added to a final concentration of 1M. after standing for 20 h at room temperature (22°C), the solution was adjusted to pH 2.5 and extracted with ethyl acetate (EA-2). The other portion was treated with pectinase (Sigma, P5146 from *Aspergillus niger*) for 10 h, adjusted to pH 2.5 and extracted with ethyl acetate (3×100 mL) (EA-3). EA-2 and EA-3 were dried and

evaporated as described for EA-1. Part of the ethanol-insoluble residue was treated with 1 M sodium hydroxide for 6 h under nitrogen at room temperature. The mixture was neutralized with 2 M sulphuric acid and filtered and the filtrate was adjusted to pH 2.5 and extracted with ethyl acetate (EA-4) as described above. The yields of the ethyl acetate extracts which contained free (EA-1), ester-linked (EA-2) and glycosidically linked (EA-3) phenolic acids extractable with aqueous ethanol and those bound to polysaccharides and/or lignin (EA-4) are given in Table 1-4 along with the amounts of the eight identified phenolic acids.

**HPLC Analysis of Phenolic Acid Fractions**

The extracts (EA-1 to EA-4) were dissolved in 10 mL ethanol in a measuring flask. Portions (2 mL) of each were transferred to a small conical flask and evaporated to dryness by flushing with

Table 1: Analysis of EA-1 extracts of phenolic acids in *M. uniflorum*

| Phenolic acids | Ethyl acetate extract yield fraction (%) | Phenolic acid contents (mg/100 g original plant dry material) |
|---|---|---|
| *p*-hydroxy benzoic acid | 0.45 | 0.95 |
| 3,4-dihydroxy benzoic acid | 0.45 | - |
| *p*-coumaric acid | 0.45 | 2.17 |
| Caffeic acid | 0.45 | 0.54 |
| Ferulic acid | 0.45 | 0.32 |
| Vanillic acid | 0.45 | 0.11 |
| Syringic acid | 0.45 | 0.05 |
| Sinapic acid | 0.45 | - |

Table 2: Analysis of EA-2 extracts of phenolic acids in *M. uniflorum*

| Phenolic acids | Ethyl acetate extract yield fraction (%) | Phenolic acid contents (mg/100 g original plant dry material) |
|---|---|---|
| *p*-hydroxy benzoic acid | 0.35 | - |
| 3,4-dihydroxy benzoic acid | 0.35 | - |
| *p*-coumaric acid | 0.35 | 0.52 |
| Caffeic acid | 0.35 | - |
| Ferulic acid | 0.35 | 0.37 |
| Vanillic acid | 0.35 | - |
| Syringic acid | 0.35 | - |
| Sinapic acid | 0.35 | - |

Table 3: Analysis of EA-3 extracts of phenolic acids in *M. uniflorum*

| Phenolic acids | Ethyl acetate extract yield fraction (%) | Phenolic acid contents (mg/100 g original plant dry material) |
|---|---|---|
| *p*-hydroxy benzoic acid | 1.63 | 7.81 |
| 3,4-dihydroxy benzoic acid | 1.63 | 3.37 |
| *p*-coumaric acid | 1.63 | 8.95 |
| Caffeic acid | 1.63 | - |
| Ferulic acid | 1.63 | - |
| Vanillic acid | 1.63 | - |
| Syringic acid | 1.63 | 0.11 |
| Sinapic acid | 1.63 | - |

Table 4: Analysis of EA-4 extracts of phenolic acids in *M. uniflorum*

| Phenolic acids | Ethyl acetate extract yield fraction (%) | Phenolic acid contents (mg/100 g original plant dry material) |
|---|---|---|
| *p*-hydroxy benzoic acid | 0.09 | 0.69 |
| 3,4-dihydroxy benzoic acid | 0.09 | 0.07 |
| *p*-coumaric acid | 0.09 | 0.49 |
| Caffeic acid | 0.09 | - |
| Ferulic acid | 0.09 | 0.18 |
| Vanillic acid | 0.09 | - |
| Syringic acid | 0.09 | - |
| Sinapic acid | 0.09 | 0.05 |

nitrogen. The dried extracts were dissolved in the mobile phase (2 mL, 30% MeOH in 0.01M phosphate buffer, pH 2.8) containing *p*-methoxyphenyl acetic acid (0.45 mg, internal standard) and were filtered. The filtrates were analyzed by HPLC on Supelco $C_{18}$ column using 0.01 M phosphate buffer (pH 2.8): MeOH, 70:30 v/v for 15 min to 60:40 for 25 min with a flow rate of 1 mL $min^{-1}$. Compounds were identified by comparison of retention times and UV spectra with those of appropriate standards analyzed under the same conditions. Quantitative determination was performed at 320 nm for cinanamic acid derivatives (ferulic, *p*-oumaric, caffeic and sinapic acids) and 254 nm for benzoic acid derivatives (*p*-hydroxy benzoic, 3, 4-dihydroxybenzoic, vanillic and syringic acids).

## RESULTS AND DISCUSSION

It is very important to determine phenols in plants, both qualitatively and quantitatively. A number of analytical methods have been proposed for the separation and determination of phenolic compounds. Most of these protocols are based on a high performance liquid chromatography (HPLC) technique with UV spectrophotometry because derivatization is not required prior to analysis (Mattila and Kumpulanen, 2002; Justesen and Knuthsen, 2001; Merken and Beecher, 2000). Before HPLC analysis, hydrolysis of glycosides or esters was necessary, so as to determine phenolic content, since a considerable fraction is in bound form (Lee and Widmer, 1996). Extraction was performed with a mixture of 80-90% aqueous methanol or ethanol. They have a protective role and can prevent phenolic compounds from being oxidized by enzymes, such as phenoloxidases (Harborne, 1998). Columns employed to separate phenolics are almost exclusively reversed-phase. This system is a high resolution chromatographic technique widely used for simultaneous separation and quantification of phenolic substances.

Phenolic acids are considered as allelopathic compounds and these play important role in biological process (Chung *et al*., 2001). Like other plants, *Cajanus cajan* (Nahar *et al*., 1990), *Fissistigma rubiginosum* (Nahar *et al*., 2001), *Ficus racemosa* (Islam *et al*., 2001), *Macrotyloma uniflorum* was also found to contain several phenolic acids in varying amounts (Table 1-4).

Extractable phenolic acids were isolated in three batches as free (EA-1), ester linked (EA-2) and glycosidically bound (EA-3) (Fig. 1) from aerial part of *M. uniflorum* plant. The latter two batches were released, respectively by alkali and pectinase treatment. Non-extractable bound phenolic acids (EA-4) were isolated by extraction of the ethanol-insoluble plant residue with alkali. The extractable glycosidically linked fraction EA-3 contain highest amount of phenolic acids among the other three fractions. *p*-Coumaric acid was the major component followed by *p*-hydroxy benzoic acid of that fraction. The non-extractable bound phenolic acid fraction (EA-4) contained five different phenolic acids namely 3, 4-dihydroxy benzoic, *p*-hydroxy benzoic, *p*-coumaric, ferulic and sinapic acids. *p*-Hydroxy benzoic acid (0.69 mg/100 g of plant dry material) was the major component of EA-4 (Table 4). Small amount of 3,4-dihydroxy benzoic acid was found only in bound phenolic acid fraction EA-4. 3,4-Dihydroxy benzoic, *p*-hydroxy benzoic, *p*-coumaric and syringic acids were found to present in the extractable glycosidically linked phenolic acid fraction, EA-3 whereas, *p*-coumaric acid (8.95 mg/100 g of plant dry material) was the major component and syringic acid (0.11 mg/100 g of plant dry material) was the minor component of EA-3. Extractable ester linked fraction, EA-2 contain only two phenolic acids, *p*-coumaric- and ferulic acids whereas amount of *p*-coumaric acid was higher than ferulic acid. Free phenolic acid fraction, EA-1 contained six phenolic acids namely, *p*-hydroxy benzoic, *p*-coumaric, caffeic, ferulic, vanillic and syringic acids. But *p*-coumaric acid (2.17 mg/100 g of plant dry material) was the major and syringic acid (0.05 mg/100 g of plant dry material) was the minor component of EA-1 fraction. Vanillic and caffeic acids were absent in all the fractions except EA-1.

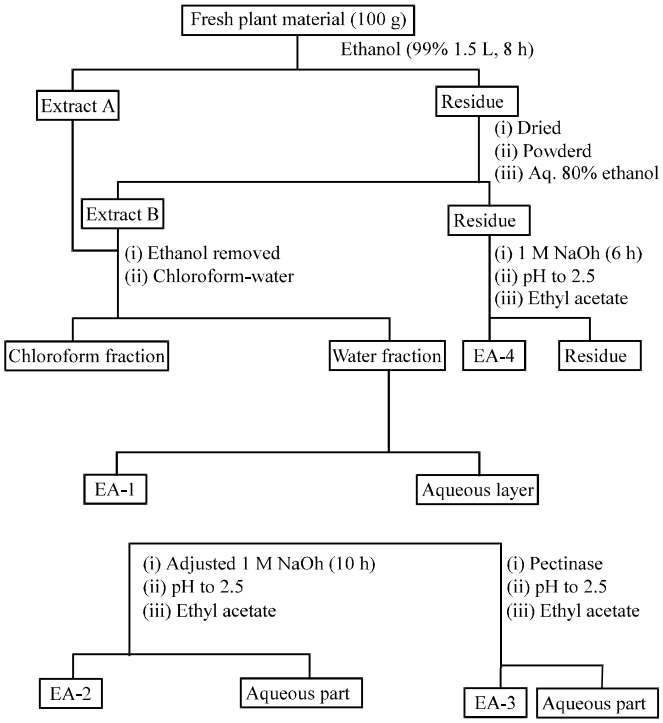


Fig. 1: Fractionation scheme of extraction and isolation of plant material

The identified and quantified phenolic acids displayed in Fig. 2-3 shows the structures and chromatogram of phenolic acids, respectively. A good resolution, with sharp peaks was achieved for all the phenolic compounds within 20 min when the solvent system and the chromatographic conditions reported in the materials and methods section were employed. Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids. Phenolic acids are a major class of phenolic compounds, widely occurring in the plant kingdom especially in fruits and vegetables. Selected phenolics in several species, separated and identified by the RP-HPLC method are shown in Table 1-4. Considerable variation was found in phenolic compounds of different species. Because of the diversity and complexity of the natural mixtures of phenolic compounds in hundreds of herb extracts, it is rather difficult to characterize every compound and elucidate its structure, but it is not difficult to identify major groups and important aglycones of phenolic compounds. Many medical herbs and species have been studied and to some extent their phenolic chemistry is known (Cai *et al*., 2004).

Antioxidant activity of *M. uniflorum* is a result of phenolic acid, especially caffeic and *p*-coumaric, acid content. The 3,4-position of dihydroxylation on the phenolic ring in caffeic acid showed increased antioxidant activity as compared to *p*-coumaric acid (Kim and Lee, 2004). Caffeic acid is expected to have higher antioxidant activity because of additional conjugation in the propenoic side chain, which might facilitate the electron delocalization, by resonance, between the aromatic ring and propenoic group.

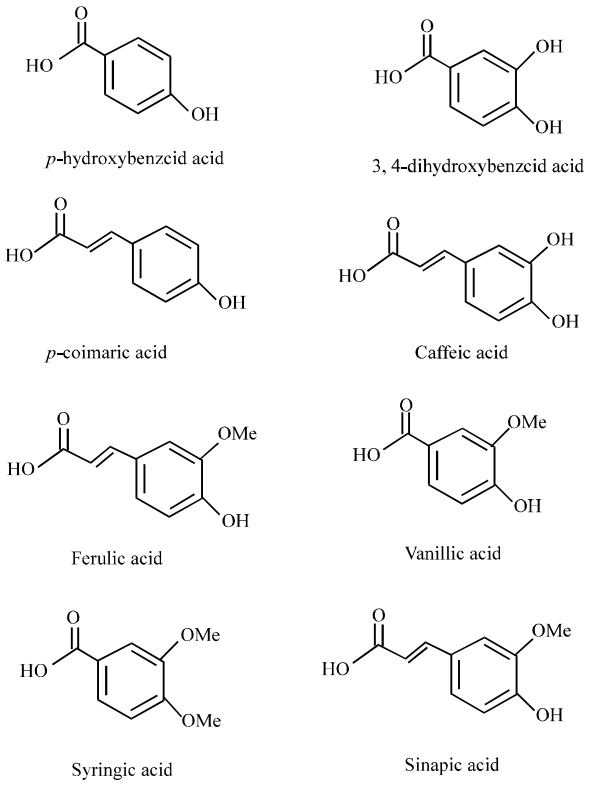


Fig. 2: Structures of the identified and quantified phenolic acids

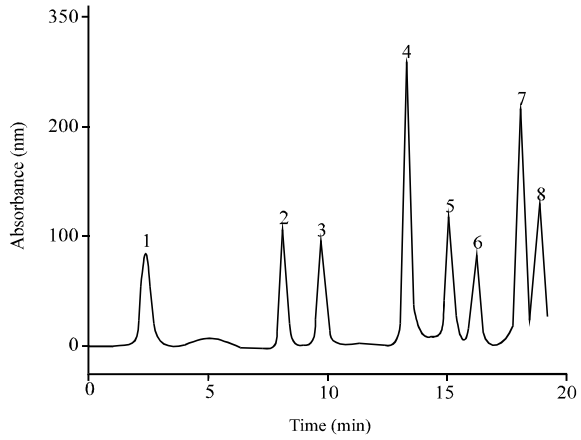


Fig. 3: HPLC separation chromatogram of phenolic acids

## CONCLUSION

The specific aims were to isolation of phenolic acids in *M. uniflorum* plant and eight phenolic acids were identified and quantified by using quantitative reversed phase HPLC method. This study shows that this plant, rich in phenolic acids could be a good source of natural antioxidants.

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