

HISTOLOGICAL AND PHYTOCHEMICAL STUDIES OF *COFFEA BENGHALENSIS* B. HEYNE EX SCHULT., COMPARED WITH *COFFEA ARABICA* L.

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Abstract

Coffea benghalensis B. Heyne ex Schult. as a wild Indian coffee species, has been rarely studied and applied in the industry nowadays. In this work, histological characters of the leaf and petiole, and polyphenolic compounds of the leaf, immature pericarp and seed were studied by LC/MS, and compared with *C. arabica* as a control species. The epidermis of the leaf and petiole of *C. benghalensis* is covered by cuticle. The leaf consists of a heterogeneous mesophyll with intercellular, palisade and spongy cells, and collateral closed bundles. These are surrounded by sclerenchyma in the petiole. Compared with Arabic coffee, the cuticle was thicker; epidermis and spongy cells smaller, and palisade cells were longer in the leaf of Bengal coffee. In the petiole, the central bundle continues into 2 small bundles in contrast to the six ones in the Arabic coffee. Five phenolic acids were identified in the leaf, immature pericarp and seed, and 4 flavonols in the leaf and pericarp. Chlorogenic acid was dominant in the non-hydrolysed seed extract, while caffeic acid in the hydrolysed extract, and isoquercitrin and rutin in the non-hydrolysed extract of the leaf. All compounds were represented at a low level in the immature pericarp. Chlorogenic, ferulic, p-coumaric and sinapic acid occur in larger amounts in the leaf and seed of *C. arabica*. Flavonols were documented at a higher level in the leaf of Bengal than of Arabic coffee. These preliminary results highlight the importance of study of coffee species.

Rezumat

Coffea benghalensis B. Heyne ex Schult. este o specie indiană sălbatică mai puțin studiată și utilizată în industrie. În prezenta lucrare au fost studiate frunza și pețiolul plantei din punct de vedere histologic, compușii polifenolici din frunză, pericarp și sămânța imatură cu ajutorul metodei LC/MS. Rezultatele obținute au fost comparate cu rezultatele înregistrate pentru specia *Coffea arabica* L. Epiderma și pețiolul speciei *C. benghalensis* sunt acoperite de cuticulă. Mezofilul heterogen al frunzei este alcătuit din parenchimul palisadic, parenchimul lacunos bogat în lacune aerifere și fascicule colaterale închise. În pețiolul frunzei, fasciculele sunt înconjurate de sclerenchim. În comparație cu specia *C. arabica*, la *C. benghalensis* s-au confirmat o cuticulă mai groasă, celule epidermice, celule spongioase mai mici și celule palisadice mai lungi. În contrast cu specia *C. arabica*, unde fasciculele mici sunt în număr de șase, fasciculul central din pețiolul frunzei de *C. benghalensis* se continuă numai cu două fascicule mai mici. Prin metoda LC/MS s-au identificat, calitativ și cantitativ, 5 acizi polifenol-carboxilici din frunza, pericarpul și sămânța de cafea și 4 flavonoli din frunza și pericarpul speciilor studiate. Acidul clorogenic a fost preponderent în extractul nehidrolizat obținut din semințe, în timp ce acidul cafeic în extractul hidrolizat, iar isoquercitrina și rutozida în extractul nehidrolizat al frunzei. Compușii identificați au fost prezenți în concentrație mai scăzută în pericarpul imatur. Acidul clorogenic, ferulic, p-cumaric și sinapic au fost prezenți în concentrații mai ridicate în frunzele și semințele speciei *C. arabica*, iar flavonolii în frunzele speciei *C. benghalensis*. Aceste rezultate preliminare subliniază importanța studiilor în cazul speciilor de cafea.

Keywords: coffee species, histology, polyphenol, LC/MS

Introduction

Coffee (*Coffea*) species originate from Africa and take the second place after petrol on the international market [4]. They belong to the *Rubiaceae* family including 450 genera and more than 6500 species [2, 18].

From all the species, the most famous is the Arabic coffee (*Coffea arabica* L.) which originates from Africa and it is cultivated in several regions worldwide [9]. The species have been studied considering the chemical, antimicrobial, pharmacological, and cosmetic aspects [14]. High concentrations of

linoleic acid of green seeds have protective effects against light because of UV absorption properties which can be used in cosmetics [17].

Bengal coffee (*C. benghalensis* B. Heyne ex Schult.), which is a wild species in India, has been reclassified into the *Psilanthus* genus (*Psilanthus benghalensis* B. Heyne ex Schult. J-F. Leroy) [10, 12]. This plant has low caffeine content [1], therefore it is rarely studied and used in the industry nowadays. In the literature, its terpenoids have been isolated and identified by various chromatographic techniques. In addition, the antimicrobial and antioxidant effect of the fruit has also been determined [8].

Coffea species are well known for their high concentration of caffeine and for polyphenolic compounds, with various chemical structures and minor sub-groups.

Although more than 100 *Coffea* species are known in the plant taxonomy, only a few taxa have been well studied nowadays. In cultivated species, the seed is the commonly investigated part of coffee species, but the leaf, stem and pericarp may provide new data by further phytochemical analyses also in cultivated and wild plants.

The aim of the present paper was to study the histological features of the leaf and petiole, and to identify and quantify polyphenolic compounds in the leaf, immature pericarp and seed of *C. benghalensis* by LC/MS to complete the available data on the species. *C. arabica* grown under similar conditions was used for each analysis as a thoroughly studied control species.

Materials and Methods

Plant materials

Samples of *Coffea benghalensis* and *C. arabica* were collected from the Botanical Garden of the University of Pécs, Hungary, in the spring of 2014. The leaf, petiole and immature fruit were air-dried at room temperature in shade. In addition, the leaf and petiole were fixed in a mixture of 96% ethanol:glycerine:water (1:1:1) for the histological investigation. Voucher specimens were deposited and labelled with unique codes at the Institute of Pharmacognosy, University of Pécs.

Sample preparation for histological study

Collected and fixed leaf and petiole of each species were dehydrated in acetone series (in 70% and 90% acetone for 1 hour each, in 100% acetone overnight), and in xylol for 2 hours in each species. Samples were kept in dissolved Paraplast at 56°C overnight. Then they were embedded in Paraplast and dried at room temperature to perform a solid sample-holder block for further section. Vertical sections were prepared by a rotary microtome (Anglia Scientific 0325). The 10 µm-thick sections were mounted on slides in water (50°C), and dried at 50°C for 2

hours. Samples were soaked in xylol for 3 x 10 minutes and in 96% ethanol for 2 x 10 minutes. Staining was performed in toluidine blue (0.02 %) for 5 minutes. Slides were covered with Canada balsam, and then studied by Nikon Eclipse 80i microscope and SPOT BASIC v4.0 program. Morphological data were measured by Image Tool 3.00 program (20 measurements in 5 sections per studied plant parts). The measured parameters were the following: thickness of cuticle, height of epidermis cells, palisade and spongy parenchyma, and width of vascular bundle in the leaf and petiole (µm), which were disposed according to the measured data of earlier studies [13].

Statistical analysis

The measured features were compared and evaluated with Two-Sample T-test. The normality of data series was checked by Shapiro-Wilk test. Differences were considered statistically significant at $p \leq 0.05$. All statistical data were calculated using Past statistic software [6].

Sample preparation for LC/MS analysis

2.5 g of dried and ground leaf, immature pericarp and seed were extracted with 5% ethanol (47.5 mL). Non-hydrolysed and hydrolysed samples were prepared for both species. Hydrolysis was carried out according to the following protocol: one part of extract was diluted with a 2 N HCl solution and it was kept on a water bath for 60 min at 80°C. The evaporated volume of liquid, during hydrolysis, was corrected by adding an appropriate amount of distilled water and afterwards it was injected into the chromatographic system. The reason of hydrolysis was that in general, some flavonoid aglycone or some polyphenol-carboxylic acids are bound with other compounds (glycosides, esters, etc.). So, hydrolysis will release these compounds from this form giving us more information about the chemical composition of polyphenols of the studied product.

LC/MS method

Polyphenolic compounds were determined by high performance liquid chromatography coupled with mass spectroscopy (LC/MS). This method aimed only the quantitative and qualitative comparison of polyphenols providing new chemotaxonomical data of the studied coffee species. The method is based on earlier analyses [3, 5, 7, 15, 16] to which we added some modifications. The major one is the changing of the components of mobile phase (acetic acid instead of potassium phosphate). The reason of this change is to obtain supplementary information about polyphenolic compounds. Due to the caffeic acid overlapped chlorogenic acid and the caftaric acid overlapped gentisic acid, they were determined in various analysis using LC/MS/MS, too.

Chromatographic separations were performed on an Agilent 1100 HPLC Series system (Agilent, USA) equipped with degasser, binary gradient pump,

column thermostat, autosampler, and UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18, 100 x 3.0 mm i.d., 3.5 μ m particle). The work temperature was 48°C. The detection of the compounds was carried out on both UV and MS modes. The UV detector was set at 330 nm until 17.5 min, then at 370 nm. The MS system operated using an electrospray ion source in negative mode. The analytical data were processed using Chem Station and Data Analysis software (Agilent, USA).

The mobile phase was a binary gradient prepared from methanol and 0.1% acetic acid solution (v/v). The start was performed with 5% methanol, and then 42% methanol (until 35 min), 42% methanol (until 38 min) and 5% methanol (until 45 min) were added. Finally, the rebalancing was achieved.

Determination of polyphenols

The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol.

The UV trace was used for quantification of identified compounds of MS detection. Using the chromatographic conditions described above, the used 18 polyphenol standards were eluted in less than 35 minutes (Table I).

Table I

Retention times (t_R) for the investigated polyphenol standards

Phenolic compound	$t_R \pm SD$ (min)
Caftaric acid*	2.10 $\pm$ 0.06
Gentisic acid*	2.15 $\pm$ 0.07
Caffeic acid*	5.60 $\pm$ 0.04
Chlorogenic acid*	5.62 $\pm$ 0.05
p-coumaric acid	8.7 $\pm$ 0.08
Ferulic acid	12.2 $\pm$ 0.10
Sinapic acid	14.3 $\pm$ 0.10
Hyperoside	18.60 $\pm$ 0.12
Isoquercitrin	19.60 $\pm$ 0.10
Rutoside	20.20 $\pm$ 0.15
Myricetin	20.70 $\pm$ 0.06
Fisetin	22.60 $\pm$ 0.15
Quercitrin	23.00 $\pm$ 0.13
Quercetin	26.80 $\pm$ 0.15
Patuletine	28.70 $\pm$ 0.12
Luteolin	29.10 $\pm$ 0.19
Kaempferol	31.60 $\pm$ 0.17
Apigenin	33.10 $\pm$ 0.15

Abbreviation: * overlapping in UV detection, only qualitative analysis possible using MS detection

Results and Discussion

Histological features

The leaf and petiole are covered by a thin cuticle on the one-cell-layer epidermis on the adaxial and abaxial surfaces in Bengal coffee (Figure 1a).

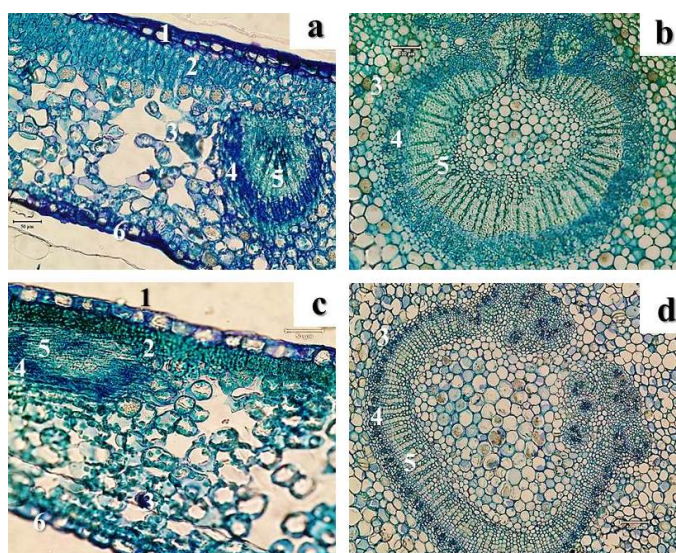


Figure 1.

Histological features of the leaf and petiole of the studied coffee species.

a. leaf of *Coffea benghalensis*; **b.** petiole of *C. benghalensis*; **c.** leaf of *C. arabica*; **d.** petiole of *C. arabica*.

In each photo: 1. adaxial epidermis, 2. palisade parenchyma, 3. spongy parenchyma, 4. phloem, 5. xylem, 6. abaxial epidermis.

The epidermis of the leaf of *C. arabica* is also one-cell-layer covered by cuticle (Figure 1c) similarly to earlier reported data [11]. In this work, the cuticle and wall of epidermis cells in sun leaves

tend to be smaller, but thicker than in shade leaves of Arabic coffee.

Among the isodiametric epidermis cells on both sides of dorsoventrally leaves of Bengal coffee, in the heterogeneous mesophyll, the elongated

palisade cells extend towards the adaxial side and isodiametric spongy cells to the abaxial surface. Palisade cells including chloroplasts extend in two layers forming chlorenchyma similarly to the earlier published features of the mesophyll in Arabic coffee [11]. In both species, several intercellular spaces can be found among the spongy cells. Stomata are in mesomorphic position at the same level with the abaxial epidermis cells in both species. The vascular bundles of each plant consist of the elements of phloem and xylem composing a collateral closed structure without cambium (Figures 1a and 1c).

In the petiole of Bengal coffee, isodiametric parenchyma cells and sclerenchymatous cells surround the collateral closed bundle in the middle of the petiole, which consist of phloem and xylem elements, similarly to the vascular bundles of the leaf (Figure 1b). The central bundle is circular

closing on the adaxial surface of the petiole, and it continues in two smaller bundles towards this side, while those of Arabic species in 6 smaller ones (Figure 1d).

Compared to the measured histological values of leaves, cuticle was thicker on both sides in Bengal than in Arabic coffee (cuticle of upper epidermis: $F = 8.27$, $t = -8.83$, $p < 0.001$); cuticle of lower epidermis: $F = 1.81$, $t = -5.99$, $p < 0.001$). Height and width of epidermis cells are smaller on both leaf surfaces of *C. benghalensis* than in *C. arabica* (height: $F = 1.68$, $t = 5.07$, $p < 0.001$, width: $F = 1.09$, $t = 2.02$, $p < 0.05$). The palisade cells are longer ($F = 11.63$, $t = -10.18$, $p < 0.001$) and wider ($F = 3.16$, $t = -3.22$, $p < 0.01$) in *C. benghalensis* than in *C. arabica*, but the values of spongy cells are converse. The ratio of the layers of palisade and spongy cells was 1:3 in Bengal coffee while 1:7 in Arabic species (Figure 2).

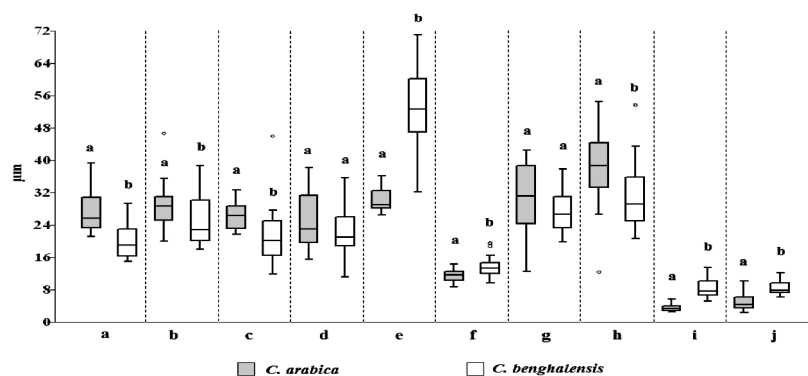


Figure 2.

Comparison of the measured histological characters of the leaf of *C. benghalensis* and *C. arabica*.

a. height of adaxial epidermis cells; **b.** width of adaxial epidermis cells; **c.** height of abaxial epidermis cells; **d.** width of abaxial epidermis cells; **e.** height of palisade parenchyma cells; **f.** width of palisade parenchyma cells; **g.** height of spongy cells; **h.** width of spongy cells; **i.** thickness of adaxial cuticle; **j.** thickness of abaxial cuticle.

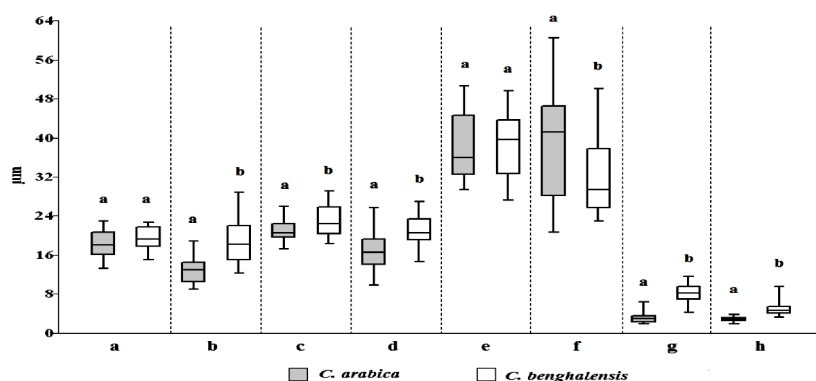


Figure 3.

Comparison of the measured histological characters of the petiole of *C. benghalensis* and *C. arabica*.

a. height of adaxial epidermis cells; **b.** width of adaxial epidermis cells; **c.** height of abaxial epidermis cells; **d.** width of abaxial epidermis cells; **e.** height of parenchyma cells; **f.** width of parenchyma cells; **g.** thickness of adaxial cuticle; **h.** thickness of abaxial cuticle.

In the petiole, adaxial and abaxial cuticle was thicker on both surfaces in Bengal than in Arabic coffee (adaxial: $F = 3.53$, $t = -9.57$, $p < 0.001$;

abaxial: $F = 8.12$, $t = -6.25$, $p < 0.001$), similarly to the values of the leaf of the plants. The isodiametric epidermis cells were longer ($F = 1.85$, $t = -2.61$, $p < 0.05$)

and wider ($F = 1.38$, $t = -3.26$, $p < 0.01$) on the abaxial side in Bengal than in Arabic coffee. The height of the isodiametric parenchyma cells was similar in both species, while they were wider in Arabic coffee than in the Bengal species ($F = 1.92$, $t = 2.31$, $p < 0.05$) (Figure 3).

Detection and evaluation of polyphenols

Nine polyphenolic compounds involving 5 phenolic acids (caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid and sinapic acid), and 4 flavonols (quercetin, isoquercitrin, kaempferol and rutin) were identified and quantified in *Coffea benghalensis* (Tables II-IV). Phenolic acids were identified also in the leaf, pericarp and seed, but flavonols appeared only in the leaf and immature pericarp of the species.

Some differences were found in the non-hydrolysed and hydrolysed extracts of Bengal coffee. In the non-hydrolysed extracts, chlorogenic acid was the dominant compound in the immature seed, while rutin was identified as a least component in the immature pericarp. In the hydrolysed extracts, a large amount of caffeic acid was detected in the leaf and kaempferol at a low level in the immature pericarp. Phenolic acids such as caffeic and ferulic acids were observed in the largest amount in the hydrolysed leaf extract, as well as in the non-hydrolysed (chlorogenic acid) and hydrolysed extracts (p-coumaric acid) of the immature seed (Tables II-IV).

Table II

Polyphenolic compounds determined in the leaf of *C. benghalensis* and *C. arabica* by LC/MS

Proposed compound and molecular formula	UV identification	<i>Coffea benghalensis</i>		<i>Coffea arabica</i>	
		Non-hydrolysed extract ($\mu\text{g/mL}$)	Hydrolysed extract ($\mu\text{g/mL}$)	Non-hydrolysed extract ($\mu\text{g/mL}$)	Hydrolysed extract ($\mu\text{g/mL}$)
Caffeic acid	–	2.26	125.55	1.82	86.52
Chlorogenic acid	–	428.73	-	605.65	-
p-coumaric acid	+	-	0.802	0.862	1.103
Isoquercitrin	+	7.439	-	6.361	-
Rutin	+	12.881	-	1.599	-
Ferulic acid	+	-	8.495	-	103.898
Sinapic acid	+	-	7.344	-	8.206
Quercetin	+	-	42.732	-	11.956
Kaempferol	+	-	5.722	-	1.544

Abbreviations: compounds were identified (+) or not (-) by UV spectrometry

Among the identified flavonols, isoquercitrin and rutin were detected as dominant compounds in the non-hydrolysed extract of the leaf, while quercetin and kaempferol in hydrolysed ones. Each selected compound was represented at a low level in the immature pericarp (Table III). In earlier reports, according to the protective role of polyphenols, the

amounts of total phenols were the same in the leaves of fruit-bearing and non-bearing coffee species. By contrast, in our investigation, the detected lower level of polyphenols is due presumably to the influence of various environmental factors to the immature pericarp which were not studied in this work.

Table III

Polyphenolic compounds determined in the immature pericarp of *C. benghalensis* and *C. arabica* by LC/MS

Proposed compound and molecular formula	UV identification	<i>Coffea benghalensis</i>		<i>Coffea arabica</i>	
		Non-hydrolysed extract ($\mu\text{g/mL}$)	Hydrolysed extract ($\mu\text{g/mL}$)	Non-hydrolysed extract ($\mu\text{g/mL}$)	Hydrolysed extract ($\mu\text{g/mL}$)
Caffeic acid	-	1.89	49.11	1.46	41.22
Chlorogenic acid	-	211.68	-	297.82	-
Isoquercitrin	+	6.823	-	7.285	-
Rutin	+	0.412	-	1.154	-
p-coumaric acid	-	-	0.266	-	0.561
Ferulic acid	-	-	1.619	-	3.237
Quercetin	+	-	1.550	-	1.550
Kaempferol	+	-	0.224	-	0.250
Sinapic acid	+	-	0.498	-	-

Abbreviations: compounds were identified (+) or not (-) by UV spectrometry

Comparing the identified and quantified polyphenols of *C. benghalensis* and *C. arabica* (Table II-IV), some differences were found in the values of chlorogenic, ferulic, p-coumaric and sinapic acid,

which occur in greater amounts in the leaf and seed of Arabic than in Bengal coffee, however, sinapic acid was observed only in the leaf of Arabic coffee.

Table IV

Polyphenolic compounds determined in the immature seed of <i>C. benghalensis</i> and <i>C. arabica</i> by LC/MS					
Proposed compound and molecular formula	UV identification	<i>Coffea benghalensis</i>		<i>Coffea arabica</i>	
		Non-hydrolysed extract (µg/mL)	Hydrolysed extract (µg/mL)	Non-hydrolysed extract (µg/mL)	Hydrolysed extract (µg/mL)
Caffeic acid	-	1.45	115.59	2.49	183.94
Chlorogenic acid	-	477.99	-	1741.00	-
p-cumaric acid	+	-	1.284	-	2.307
Ferulic acid	+	-	8.495	-	11.174
Sinapic acid	+	-	0.660	-	-

Abbreviations: compounds were identified (+) or not (-) by UV spectrometry

Conclusions

The detected histological features and polyphenolic compounds of *Coffea benghalensis* add new scientific data to the available records of the species. Compared with the known data of *C. arabica*, our results confirm the possible role of sinapic acid as chemotaxonomic marker for the identification of the selected species. Based on these evidences, these phytochemical results indicate that in addition to the seeds, other plant parts could be used in medicine and cosmetic industry.

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