HISTOLOGICAL, PHYTOCHEMICAL AND ANTIMICROBIAL EVALUATION OF *COFFEA* SPECIES

Ph.D. dissertation

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1. Introduction

Coffea species are well-known and widespread all over the world. They have an important role in science because of their pharmacological role. They provide one of the most sought products after petrol on the international market, and they also provide an income for more than 20 million families in more than 50 countries every year (DAVIS et al. 2007, PATAY et al. 2016c). In addition, coffee is one of the most widely consumed beverages worldwide with an annual consumption rate of approximately 7 million tons according to Food and Agriculture Organisation of the United Nations (FAO) (BAEZA et al. 2014). However, the most famous species are *C. arabica*, *C. robusta*, and *C. liberica*, we can also find scientific data on wild coffee species and subspecies cultivated in almost all continents, but these data are insufficient in the recent literature (PATAY et al. 2014a).

Due to their significant in caffeine and polyphenol content, *Coffea* extracts possess numerous physiological effects like activity on the central nervous system, as well as antioxidant, anticancer, gastrointestinal, cardiovascular, antibacterial, and dermatological effects. However, there are many previously reported scientific data about these beneficial effects for human body, an inadequate utilization of coffee can cause serious secondary effects.

According to the previously mentioned great impact of coffee on scientific and social field, the specific aims of this study were the followings:

- to realise a comparative histological, phytochemical and anitmicrobial analysis of three *Coffea* species: two well know species namely *C. arabica* and *C. liberica*, and a less studied wild species namely *C. benghalensis*.
- to complement the insufficient scientific data of *C. benghalensis* in the mentioned aspects, to emphasize its importance and to find new sources of natural antioxidants for nutraceuticals, as well as new utilization of wasted residues of coffee products
- to observe, to compare and to measure the histological parameters of the leaf, the petiole, and the fruit of the selected *Coffea* species
- to investigate the phytochemical variations, to identify and quantify some polyphenolic compounds of the leaf, the immature and mature fruit of the selected coffees by HPLC methods
- to compare the polyphenol, tannin and total flavonoid content of the selected coffees

- to investigate the antioxidant activity of the selected *Coffea* species by Enhanced Chemiluminescence (ECL), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Oxygen Radical Absorbance Capacity (ORAC) assays
- to prove the antimicrobial effects of each coffee extract with disc and agar diffusion against various microorganisms.

2. Literature review

2.1. Description and synonyms of coffee names

Based on the significant role of coffee as the most consumed beverage in the world, several names become popular and known in the plant terminology in various languages. In Hungarian the word "*kávé"* means coffee and it may have an Ottoman-Turkish (*kahve)* (RÁCZ 2010) or Arabic-Turkish *(kahwe, quahwa*) origin. It may mean an Ethiopian province named „*Kaffa"* (BRÜCHER 1977), which was also transferred from Arabic to Turkish terminology (*arab gahwa*). In Hungarian there are many coffee expressions like *mokkakávé* which refers to port city called Mokka in Jemen (RÁCZ 2010).

The most frequently used name for coffee is „*kávé"* which refers to the Arabic coffee (*C. arabica)* as far as botany is concerned. In addition, there are a lot of international names for these plants, e.g.: *akeita* or *café* (France), *araabia kohvipuu* or *kohv* (Estonia), *cafea* (Romania), *caffe* (Finland, Italy), *kahvi* (Finland), *kafa* (Serbia, Albania, Bulgaria, Czech Republic, Gambia), *kafe* (Greece), *kaffee* (Denmark, Sweden), *kaffe* or *kaffeeplante* (Norway), *Kaffeestrauch* (Germany), *kaffi* (Iceland), *kafija* (Latvia), *kahva* (Bosnia), *kahve* (Turkey), *kawa* (Poland), *kofe* (Russia), *koffie, koffieboom* or *kofje* (Netherlands), and *kave* (Croatia, Lithuania, Slovakia, Slovenia, Ukraine). In Africa usually the names *café, koffie, kofi, ikhofi, ikofu* or *kahawa* are used, while it is known as *bunna* in Ethiopia. In Senegal and Latin America the word *kafe* is known, but *kahioa* or *qahwah* in Arabic countries, *ghah'veh* in Iran, *ka'fe or kave* in Israel, *ka-fae* or *gafae* in Thailand, *ga Feh* or *ka-fei* in China, *kaafi, koffee* or *kofii* in India, *koohii* in Japan, *kopi* in Indonesia and Malaysia, *ko-pi* in Sri Lanka, *ko-pyi* in Korea, *qahve* in Azerbaijan and Yemen, *sourdj* in Armenia, *kaapi* in Mexico and Central America, *kaawa* in Uganda, *café* in Argentina, Bolivia, Brazil, Catalonia, Chile, Ecuador, Peru, Portugal, Spain, and Vietnam, *chai* in Georgia, *coffee* or *koffi* in Guyana and the United Kingdom, *coffee* or *lee-cah fee* in the United States, *kape* in the Philippines, as well as *kope* are widely used in the Hawaiian islands (ROSS 2005).

2.2. Historical description and discovery of *Coffea* species

In Europe the Botanical Garden of Amsterdam was the first place which had a coffee plant in 1710. The first botanical description has been prepared by A. De Jussieu (1713), who has investigated a plant from this botanical garden. Carl Linné reclassified this species to another

group in 1753 and he named it *Coffea arabica* which was the single known coffee species at that time (CLIFFORD and WILLSON 1985).

Many legends are known regarding the discovery of coffee seeds due to its widespread utilization and pharmacological effects over the years. Local people living in East Africa have eaten the raw red coffee beans before tribal wars and hunts. Based on the most famous historical data, some Coptic monks or some Ethiopian goat herders discovered the stimulating effects of the seeds due to their goats which were more energetic after eating them (2^{th} century). The herders also tasted the seeds and they also felt stimulating effect. Due to this reason they have used the fermented fruits mixed with cereals and animal fat for preparing scones which were indispensible for their longer trips (BABULKA et al. 2012).

The most common story tells about a shepherd named Khaldi, who lived in the 15^{th} century and he also observed the stimulating effect of coffee seeds. He shared his observations with monks who have prepared a boiled beverage used the seeds. They used to drink it during their long ceremonies (CROZIER et al. 2012).

Another memory tells about Dzsemal-Eddin, who was a mufti of Aden. When he travelled to the West coast of the Red Sea around 1450, he drank a dark beverage in a weak health condition. Because he liked this drink, he began to drink it regularly (RÁCZ 2010).

For a long time the Arabic countries used to prohibit the consumption of coffee among men by referring to the Koran. People in Constantinople established for men special places called "Mektab-i-irfan" in the 16^{th} century, where Turkish dignitaries were served by coffee. First time only the dignitaries were served, but later coffee became more popular, therefore other places were opened to attend the lower classes, too. As Turkish men spent a lot of time in these establishments, the mosques began to be abandoned, so imams opposed coffee consumption, which was forbidden by a law ordered by Muhammad. Due to this reason Sultan Murad banned coffee consumption, but this prohibition was applied only to men because as far as religion was concerned that women can not go to Paradise after their death. Later, when this prohibition was modified when the use of tobacco began to become more popular. It is supported by the following Turkish proverb: *"Coffee without tobacco is like food without salt."* (RÁCZ 2010).

A doctor from Ausburg, named Leonhard Rauwolf, wrote the following words about coffee*: "It is black as the ink but its good for the stomach."* Prosper Alpino, a teacher from Padua, prepared the botanical description of the plant in 1592. In his work involving data of Egyptian plants he

mentioned coffee plant as "*Arbor bon cum fructu suo buna"* in which coffee may have been named "bun" (RÁCZ 2010).

Coffee was also mentioned as a hot drink in the Baroque Era written by Miklós Zrínyi in 1651. In the third part of *Szigeti Veszedelem* the following sentences can be read: "*Szkender, ha akarod, ketten együtt háljunk. Meleg kávé mellett agg szót kovácsoljunk!"* (RÁCZ 2010)

Coffee as beverage has been used in Europe since the 17th century used the roasted and powdered seeds (KOTHE 2008). Although the first coffee house has been opened in Venice in 1624, based on other data the beverage has been known in Hungary even earlier. According to two Hungarian legends, Bálint Török had a famous dialogue with Sultan Suleiman who organised a feast celebrating after the occupation of Buda on 29th August in 1541. He told to Bálint Török the following: "*Hátra van még a feketeleves*." After this conversation Bálint Török was taken to Constantinople and he was imprisoned in the Jedikula. A similar dialogue has been documented in the second legend between the count Imre Thököly and Ahmed Pasha (RÁCZ 2010).

According to historical data, when Romanians occupied Ajud in 1849, they took coffee seeds like the beans used by the aristocracy. However, as they could not be boiled properly, they found them useless. For this reason, harvested seeds were sold at the market in Alba Iulia (RÁCZ 2010).

Although this beverage has been known from the earlier times, Hungarians did not like it either in the 17th century. Hungarian writers named it ironically "coffee soupe" or "the black" in 1813. In the past, the drink has been used as a painkiller for stomachache, indigestion, bloating, and hangover in the country.

The first coffee house was opened in Bratislava in Hungary in 1730, and the most famous one was the Pilvax coffee house in Budapest in 1848. Documents mentioned institutions from 1706 to 1709 called *"kávésház"* and *"kaféház"*. Due to many coffee houses in Budapest, the town was transformed into the city of coffee houses by 1900 (RÁCZ 2010). During this period Guatemala was considered as a nation where coffe played an important role in the everyday life of people, e.g. they inserted coffee branches into the crest of the country (RÁCZ 2010).

In Europe, 1.4 million quintals of coffee were consumed in 1820, 2.26 million in 1840, and 6.5 million in 1865. It is an interesting data that the word for reward is *"Trinkgeld"* in Germany (drink money), "*borravaló*" in Hungary, "*tubákpénz*" in Spain and Portugal, and *"baksis"* (coffee money) in Turkey (RÁCZ 2010).

Africa provided 1% of the total coffee production of the world until World War I which was increased to 15% in the following 50 years. In 1977, America offerred 80% of the total coffee production worldwide (BRÜCHER 1977), Arabic coffee makes up 75%, Robusta coffee 24%, while Liberian coffee 1% of the total production of the world. Nowadays in terms of economy, Arabic coffee is the most important coffee plant worldwide, which provides a salary for more than 25 million people (ROHWER 2002).

2.3. Taxonomy

Coffea species belong to the Magnoliophyta phylum, Rosophytina subphylum, Rosopsida class, Asteridae subclass, Solananae superorder, Rubiales order, Rubiaceae family, and Ixoroideae subfamily (BORHIDI 2008). Rubiaceae family is a significant one in the world as far as number of members is concerned; it includes 450 genera and more than 6500 species (WIART 2006). It is mostly made out of woody, tropical and temperate herbs, lianas, epiphytes, and myrmecophyl plants as well as the Indo-Malay root epiphytes (e.g. *Myrmecodia, Cuviera,* and *Hydnophytum* sp.) (JUDD et al. 1999). In addition, many new Mexican taxa and their combinations have been described recently by Hungarian authors, e.g. *Edithea guerrerensis*, *E. oaxacana*, *E. rupicola*, *E. serboi* and *E. sousae* were transferred from *Deppea* genus, *Houstonia mcvaughii* and *H. acerosa* ssp. *tamaulipana* from *Hedyotis* genus, *Palicourea buchtienii* from *Psychotria* genus, as well as *Randia mendozae* was noted as a new name for replacement the homonym *R. multiflora*, *Stenaria muellerae* ssp. *pooleana* and ssp. *brevipedicellata* were noted as two new status, and *Deppeopsis* sp. and *Donnellyanthus* sp. were transferred into the *Rondeletia* complex (BORHIDI et al. 2004a,b, 2011, 2012a,b).

Rubiaceae family involves trees, lians, shrubs, and herbs which have no internal phloem in any plant parts. Entire leaves are arranged in opposite or whorled position, and they have usually pinnate venation. Stipules are interpetiolar and usually connate, occasionally leaflike with colleters on the adaxial surface. Bisexual flowers can be found in radial position. They are frequently heterostylous and aggregated. The connate 4-5 sepals are wheel-shaped. The funnel-shaped corolla is pubescent on the adaxial surface; the petals are lobate and valvate, imbricate or contorted. The number of stamens is usually 4 or 5; the basally connate filaments grow to the corolla positioned within the corolla tube or at the edge. The anthers have two loculaments, which open by a longitudinal slit. Pollen grains are usually tricolporate. The number of the connate carpels varies

from 2 to 5. The ovary can be found in inferior position with axile placentation. Stigma (1-2) is linear, capitate or lobed. The number of the ovules varies from one to numerous in each locule; they have one integument and a thin-walled megasporangium. Nectar disc presents generally above the ovary. Fruit is a loculicidal or septicidal capsule, berry, drupe, schizocarp or indehiscent pod. Seeds are sometimes winged, the embryo is straight to curved, some of them can be observed with or without endosperm (JUDD et al. 1999).

Nowadays, 128 *Coffea* species are mentioned with several varieties in scientific reports (Table 1-3). The wild species *C. benghalensis* (Bengal coffee) has been recently reclassified into the *Psilanthus* genus named *P. benghalensis*, whereas other *Psilanthus* species have been transferred to *Coffea* genus, like *P. mannii* (DAVIS et al. 2011a, [1]http, STRANCZINGER et al. 2014, DAVIS 2011b). *P. bababudanii*, which was transferred to *Coffea*, was recognized as a new species allied to *P. benghalensis* by Sivarajan in 1992, and it was placed into the synonyms of *P. benghalensis* by Deb in 2002 (DAVIS 2010).

Table 1. **Synonyms and variations of *Coffea arabica* L.** ([1]http)

Coffea species	*Synonyms*	*Variations*
***Coffea arabica* L.**		*C. arabica* var. *amarelle* A. Froehner
		C. arabica var. *angustifolia* Cramer
		C. arabica var. *bourbon* Rodr. ex Choussy
		C. arabica var. *brevistipulata* Cif.
		C. arabica var. *bullata* Cramer
		C. arabica var. *columnaris* Ottol. ex Cramer
		C. arabica var. *erecta* Ottol. ex Cramer
		C. arabica var. *humblotiana* (Baill.) A.Froehner
		C. arabica var. *intermedia* A. Froehner
		C. arabica var. *longistipulata* Cif.
		C. arabica var. *maragogype* A. Froehner
		C. arabica var. *mauritiana* (Lam.) Willd.
		C. arabica var. *mokka* Cramer
		C. arabica var. *monosperma* Ottol. & Cramer
		C. arabica var. *murta* Lalière
		C. arabica var. *pendula* Cramer
		C. arabica var. *polysperma* Burck
		C. arabica var. *pubescens* Cif.
		C. arabica var. *purpurascens* Cramer
		C. arabica var. *rachiformis* (Baill.) A.Froehner
		C. arabica var. *rotundifolia* Ottol. ex Cramer
		C. arabica var. *straminea* Miq. ex A.Froehner
		C. arabica var. *stuhlmannii* A. Froehner
		C. arabica var. *sundana* (Miq.) A.Chev.
		C. arabica var. *variegate* Ottol. ex Cramer

Table 2. **Synonyms and variations of *Coffea robusta* L. Linden**. ([1]http)

Coffea species	***Synonyms***	***Variations***
***Coffea robusta* L. Linden**	*C. canephora* Pierre ex A. Froehner	*C. arabica* var. *stuhlmannii* A. Froehner *C. canephora* var. *bukobensis* A. Zimm. *C. canephora* var. *crassifolia* Lautent ex De Wild. *C. canephora* var. *gossweileri* A. Chev. *C. canephora* var. *hiernii* Pierre ex De Wild. *C. canephora* var. *hinaultii* Pierre ex De Wild. *C. canephora* var. *kouilouensis* De Wild. *C. canephora* var. *laurentii* (De Wild.) A. Chev. *C. canephora* var. *maclaudii* (A. Chev.) A. Chev. *C. canephora* var. *muniensis* Pierre ex De Wild. *C. canephora* var. *oligoneura* Pierre ex De Wild. *C. canephora* var. *opaca* Pierre ex De Wild. *C. canephora* var. *robusta* (L. Linden) A. Chev. *C. canephora* f. *Sankuruensis* De Wild. *C. canephora* var. *sankuruensis* (De Wild.) De Wild. *C. canephora* var. *stuhlmannii* (A. Froehner) A. Chev. *C. canephora* var. *trillesii* De Wild. *C. canephora* var. *ugandae* (Cramer) A. Chev. *C. canephora* var. *welwitschii* (Pierre ex De Wild.) A. Chev. *C. canephora* var. *wildemanii* Pierre ex De Wild. *C. laurentii* De Wild. *C. maclaudii* A. Chev. *C. robusta* L. Linden *C. ugandae* Cramer *C. welwitschii* Pierre ex De Wild.

Table 3. **Synonyms and variations of other *Coffea* species** ([1]http)

Coffea species	***Synonyms***	***Variations***
***Coffea liberica* Hiern**	*C. liberica var. liberica*	*C. dewevrei* (De Wild. & T. Durand) Lebrun *C. liberica* var. *liberica*
***C. benghalensis* B. Heyne ex Schult**	*P. benghalensis* (B. Heyne ex Schult.) J-F. Leroy *C. benghalensis* var. *benghalensis* *C. floreifoliosa* A.Chev. *C. semiexserta* Colebr. ex Roxb.	*Psilanthus bababudanii*= *C. benghalensis* var. *bababudanii* (Sivar., Biju & P. Mathew) A. P. Davis
***Psilanthus mannii* Hook. f.**	*C. mannii* Hook. f.) A. P. Davis comb. nov. *C. gilgiana* A. Froehner *P. comoensis* Pierre ex De Wild. *P. ledermannii* A.Chev. *P. tetramerus* Hiern	
***Coffea congensis* A. Froehner**		*C. congensis* var. *chalotii* Pierre ex De Wild. *C. congensis* var. *froehneri* Pierre ex De Wild. *C. congensis* var. *oubanghensis* Pierre ex De Wild. *C. congensis* var. *subsessilis* De Wild.
***Coffea kapakata (*A. Chev) Bridson**	*P. kapakata* A.Chev.	

Table 3. **continued**		
Coffea species	***Synonyms***	***Variations***
***Coffea zanguebariae* Lour.**	*Amaioua africana* Spreng. *Amajoua africana* Spreng. *C. ibo* A.Froehner *C. schumanniana* Busse *Hexepta axillaris* Raf.	
***Coffea racemosa* Lour.**		
***Coffea ligustroides* S. Moore**		
***Coffea mauritiana* Lam.**		*C. mauritiana* var. *lanceolata* A.Chev.
***Coffea charrieriana* Stoff. & F. Anthony**		
***Coffea excelsa* A. Chev.**	*C. liberica* var. *dewevrei* (De Wild. & T. Durand) Lebrun)	
***Coffea brevipes* Hiern.**		*C. brevipes* var. *longifolia* A.Froehner
***Coffea stenophylla* G. Don**		*C. stenophylla* var. *camaya* Portères
***Coffea eugenioides* S. Moore**		*C. eugenioides* var. *kivuensis* (Lebrun) A.Chev.
***Coffea heterocalyx* Stoff.**		
***Coffea anthonyi* Stoff. & F. Anthony**		
***Psilanthus travancorensis* (Wight & Arn.) J.-F.Leroy**	*C. travancorensis* Wight & Arn.	
***Coffea wightiana* Wall. ex Wight & Arn.**	*Psilanthus wightianus* (Wall. ex Wight & Arn.) J.-F.Leroy	
***Coffea lebruniana* Germ. & Kesler**	*Psilanthus lebrunianus* (Germ. & Kesler) J.-F.Leroy ex Bridson	
***Coffea sessiliflora* Bridson**	*Coffea sessiliflora* subsp. *sessiliflora*	
***Coffea resinosa* Hook. f. Radlk.**	*Buseria resinosa* (Hook.f.) T.Durand ex K.Schum. *Leiochilus resinosus* Hook.f.	*Coffea resinosa* var. *thouarsii* A.Chev.
***Coffea leroyi* A. P. Davis**		
***Coffea humilis* A. Chev**		
***Coffea salvatrix* Swynn. & Philipson**		
***Coffea heterocalyx* Stoff.,**		
***Coffea humbertii* J.-F. Leroy**	*Paracoffea humbertii* J.-F. Leroy	
***Coffea vatovavyensis* J.-F. Leroy**		
***Coffea pseudozanguebariae* Bridson**		

2.4. Geographical distribution

Coffea taxa grow in tropical and subtropical areas, especially in the Equatorial region at an altitude of 200-1200 m and at 18-22°C, but a range from 1,300 to 1,600 m a.s.l. was identified as an optimum altitude for wild coffees (ROHWER 2002, TESFAYE et al. 2013). Coffee shrubs, which are

cultivated at higher regions, are named "high grown" coffee whose quality is outstanding (MĂNDIȚĂ 2008).

Coffea arabica, which is the most widespread species among the known coffee taxa (GONZALEZ et al. 2005), is native to the southern highlands of Ethiopian mountains as well as the Boma Plateau of eastern Sudan and Mount Marsabit of Kenya. In Ethiopia, wild populations occur in mountain forests on both the western and eastern sides of the Great Rift Valley in the southern part of the country. It grows naturally in the understorey of montane rain forests at altitudes between 950 and 2,000 m a.s.l. There are four different coffee production systems: the forest coffee and the semi-forest coffee, which are based on collecting coffee from autochthonous populations, and the garden and plantation production systems, where the coffee genotypes and landraces have been selected locally by farmers, and commercial cultivars are grown and managed intensely (TESFAYE et al. 2013, DAVIS et al. 2007, PATUI et al. 2014).

With commercial purposes these shrubs are grown in many parts of the world for example in South America (Brazil, Colombia, Venezuela, Bolivia, Peru, Ecuador), Central America (Mexico, El Salvador, Cuba, Haiti, Dominica, Nicaragua, Guatemala), Africa (Angola, Liberia, Ethiopia, Congo, Kenya, Tanzania, Uganda, Nigeria, Malawi), and Asia (India, Sri Lanka, Malaysia, Indonesia, Java, Sumatra, New Guinea) (MĂNDIȚĂ 2008, TÓTH 2010). Wild species do not occur in the American continent; native taxa are found only in Africa and in the South Asian regions (BRÜCHER 1977), In India, coffee shrubs are native to Tamil Nadu, Karnataka, and Kerala areas (KHARE 2007). The wild Bengal coffee (*C. benghalensis*) originates also from India, which is less used by the food industry, but it is more interesting for recent science (NOWAK and SCHULZ 2002). *C. kapakata* (Mozambique), *C. zanguebariae* (Zanzibar), *C. racemosa* and *C. ligustroides* (East Africa) belong to the native flora in Africa. Even though *C. racemosa* has a low caffeine content, it makes up the basis of several studies along with other coffee species (*C. congensis*, *C. dewevrei*), as far as coffee growing is concerned due to their genetic properties for example their significant drought tolerance and resistance to infections, rapid germination and embryo development (MIRIAN et al. 2006).

In addition to the abovementioned plants, many less studied native species are known in Africa, e.g. *C. mauritiana* (Madagascar), *C. dewevrei* and *C. liberica* (Liberia, Ivory Coast), and *C. brevipes* (Sierra Leone). *C. congensis, C. stenophylla*, *C. arabica* var. *bullata* and *C. eugenioides*

have been described in Ethiopia, the region of Congo, Ghana, Cameroon and Gabon, while *C. travancorensis* and *C. wightiana* have been documented in Sri Lanka (BRÜCHER 1977, [1]http).

2.5. Morphological description

Coffee species are densely or low branching shrubs or no more than 6-8 m high trees which are mostly evergreens and rarely deciduous plants.

The deciduous leaves are arranged in opposite position. The leaf blade is intact, more or less wavy-edged, oval or lance-perverse. It has obtuse tip and wedge-shaped base leading toward the summit lateral veins which prominent on the shiny dark green surface and the dull and lighter abaxial side (Fig. 1).

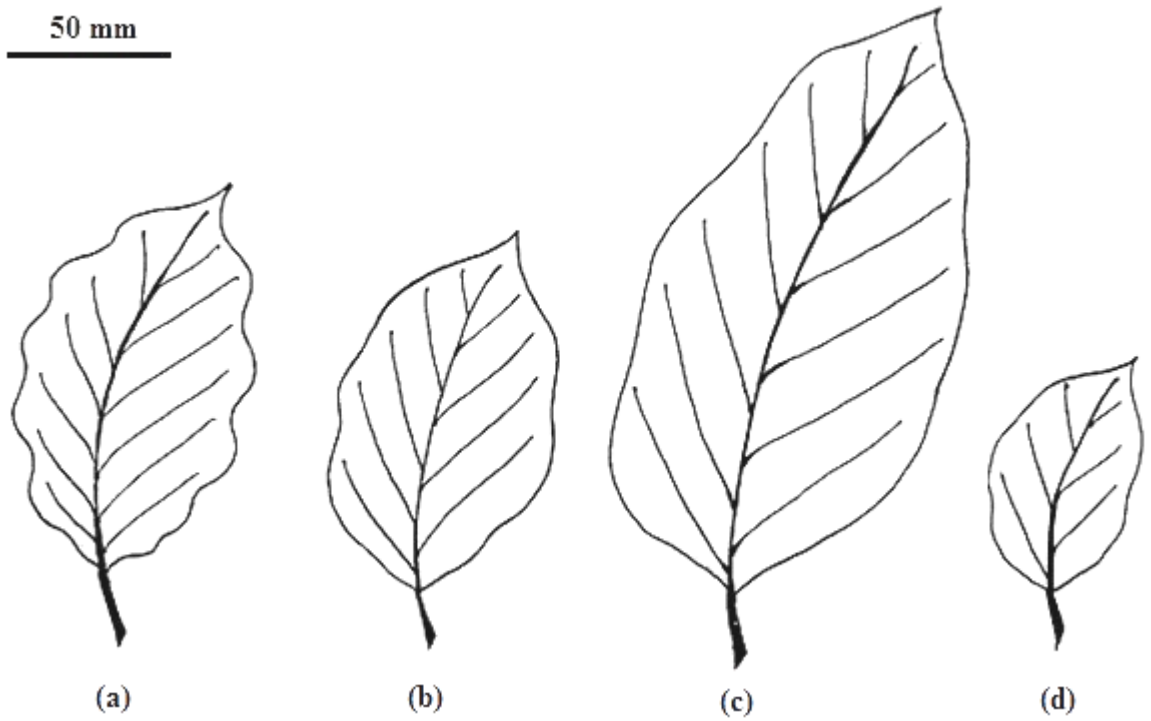

Fig. 1. Morphological comparison of the leaves of (a) *C. arabica*, (b) *C. robusta*, (c) *C. liberica*, and (d) *C. benghalensis* (drawing: Éva Brigitta Patay)

The maximum 5 cm long flowers occur in groups forming short inflorescences at the origin of the leaves. A calyx is short-barreled and ends in five wide and triangular lobes of sepals. The 5-8 membered white corolla grows into a tube in its lower half, the petals have lobes developing in whole blooming period.

The ripe stone fruits are orange-red, reddish brown or reddish black. They develop crowded at the top of short and thick peduncles, and they end in a barrel-shaped funiculus. Under the hard and shiny epicarp, a fleshy red internal part and a thin yellow glassy seed coat can be found. The 2 bone-shaped and elliptical seeds (= coffee beans) turn to each other with their flattened surface (Fig. 2). Coffee beans are surrounded by 'silverskin' as a thin membrane-like layer in the fruit.

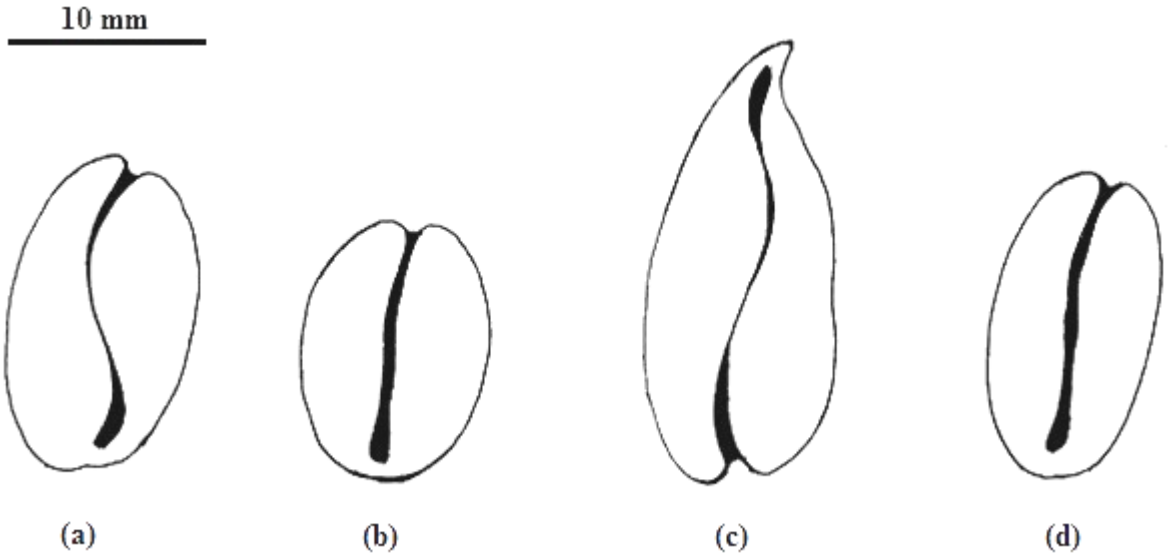

Fig. 2. Morphological comparison of the seeds of (a) *C. arabica,* (b) *C. robusta,* (c) *C. liberica,* and (d) *C. benghalensis* (drawing: Éva Brigitta Patay)

They are convex on the external side and flat on the internal side with a longitudinal sulcus. In some cases the fruits contain only one seed which is called "pearl coffee", which has higher quality owing to its intensive aroma (MĂNDIȚĂ 2008).

In *C. arabica*, the leaf is maximum 25 cm of length and 10 cm of width which is similar to those of *C. robusta*. The stone fruits of Arabic coffee fall down during the ripening period, while those of *C. robusta* coffee remain on the plant for a long time (BRIDSON and VERDCOURT 1988). The leaf bade of Robusta coffee is very light (BRIDSON and VERDCOURT 1988), and at the origin of the leaves develop even 30 flowers (BRÜCHER 1977). Both species have fruits on short peduncle which are 1.5 cm long and 1.2 cm wide.

C. benghalensis is a 2-3 m tall shrub which has oval or elliptical leaves. These can reach 3-6 cm of width and 5-7 cm of length, while the petiole can be 3-10 mm of length. The stipules are 5-6 mm

long. The flowers (1-5) are located in groups at the origin of leaves. The calyx is 3-5 mm long. The cylindrical corolla having five petals is 17-20 mm long and 3 mm wide. The elliptical stone fruits are 10-13 mm long and 5-8 mm wide (SIVARAJAN et al. 1992).

C. liberica is a 3-8 m high shrub that differs from the previous two species due to its large-sized leaves and fruits. The leaf blade can be 14-38 cm of length and 5.5 to 20.5 cm of width. The petiole is 1.5 cm long (NOWAK and SCHULZ 2002, BRIDSON and VERDCOURT 1988). The flowers are 2 cm wide having 6-8 lobes (BRÜCHER 1977). The fruits, which have a 5 cm long peduncle, can be 2.5 cm in size as well (NOWAK and SCHULZ 2002, BRIDSON and VERDCOURT 1988).

C. dewevrei, which is a less known species, has a 30 cm wide trunk, 30 cm long and 15 cm wide leaves and 1.5 cm long fruits. *C. congensis* can be characterized by tiny fruits compared to the previous species (BRÜCHER 1977).

2.6. Histological description

The taxonomical classification of coffees is based on histological features besides morphological and chemotaxonomical characters. There are only few histological data of coffees in relation to stomata and seed in current scientific references. It was earlier proved that stomata number is inversely proportional to the number of chromosomes in polyploid coffee taxa (BRÜCHER 1977). The seed coat investigated by electron microscopy has pitted thick cell wall of elongated and spindle-shaped stone cells (sclereids) which occur individually or in groups. The major part of the seed is filled with endosperm which has cells with thick cell wall. The small embryo is situated at one of the poles of the seed (SZŐKE and KÉRY 2003).

2.7. Molecular biological characters

The most frequent basic genome of Rubiaceae family is x = 11. Many studied *Coffea* and *Psilanthus* species are diploids having 2n = 22 chromosomes, like *C. liberica, C. robusta, C. kapakata, C. zanguebariae, C. racemosa, C. ligustroides, C. mauritiana, C. dewevrei, C. excelsa, C. brevipes, C. congensis, C. stenophylla,* and *C. eugenioides*. The most species are self-incompatible except the tetraploid *C. arabica* (2n=44) (BRÜCHER 1977), and the diploid *C. heterocalyx* Stoff. and *C. anthonyi* Stoff. & F. Anthony which are self-compatible (ANTHONY et al. 2010).

According to molecular markers, coffee trees are divided in two sections namely Mascarocoffea and Eucoffea. The latter contains five sub-sections: Erythrocoffea (including *C. arabica* and *C. canephora*), Pachycoffea (incl. *C. liberica*), Nanacoffea, Melanocoffea, and Mozambicoffea. *C. liberica* has two known varieties namely *C. liberica* var. *liberica* (Western Africa) and *C. liberica* var. *dewevrei* (Central Africa), which can be genetically distinguished and evaluated based on morphological traits, molecular markers, and male fertility of F1 hybrids (N'DIAYE et al. 2005).

C. arabica var. *bullata* has been described with 6n=66 chromosomes (BRÜCHER 1977). Differences from the normal chromosome number have been rarely described, but specimens of *C. arabica* have been observed in Sybenga in 1960 as triploid (3n=33), pentaploid (5n=55), hexaploid (6n=66), and octoploid (8n=88) plants. There are a few haploid or di-haploid species (monosperma plants) among young plants, which usually have narrower leaves than the other plants (CLIFFORD and WILLSON 1985). The morphology of coffee chromosomes was examinated firstly by Mendes and Bouharmont. The number and length of chromosomes of *C. benghalensis* was studied also by J. Bouharmont in 1963, who found 22 somatic chromosomes with 28.44 µ (average of data of 10 cells) of length measured in methaphase (BOUHARMONT 1963). Due to these data and the technological evolution, scientists identified that the chromosome size can vary between 1-3 microns in coffees (CLIFFORD and WILLSON 1985).

Since *C. arabica* is the most studied coffee species with the biggest economical importance, plant regeneration protocol via direct somatic embryogenesis of *C. arabica* cvs. *Caturra* and *Catuaí* has been also investigated. The applied 3 protocols were Yasuda, Hatanaka, and Catie ones. The most efficient protocol inducing direct somatic embryogenesis and embryo-toplant conversion for *Caturra* variety was Yasuda protocol, whereas Hatanaka was the most suitable for *Catuaí* variety (GATICA et al. 2007).

In another study, RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeat), and SRAP (sequence related amplified polymorphism) methods were used for the identification of genetic relationship between five native coffee species in India (one of them was *C. benghalensis*) and the cultivated *C. canephora*. The results demonstrated that these three marker systems can be useful for the identification of genetic diversity and the analysis of indigenous coffees providing an important input for conservation biology (KUMAR et al. 2011).

In a cytogenetical experiment, four taxa (*C. brevipes*, *C. racemosa*, *P. benghalensis*, *P. travancorensis)* obtained from Coffee Germplasm Bank of IAC were studied. Although

remarkable cytological similarity was observed between the studied plants, the analysis based on these cytogenetic markers contributed to karyological characterization and provided more data to taxonomical discussion in the group (LOMBELLO and MAGLIO 2004).

2.8. Reproduction, cultivation, processing

In South America, the first coffee-colonization was organised in 1933. *C. arabica* propagates by self-pollination, while *C. robusta* by allogamy. Many types of coffee propagate by seeds or cutting. Among some taxa spontaneous hybridization was also observed, for example between *C. robusta* and *C. eugenoides* (BRÜCHER 1977). These plants are often cultivated both in large-scale plantations and at small holder farms. In addition, they are also planted at the base of shady trees, which protect them against the strong sunlight. The species prefer mostly the tropical climate. The Arabic coffee develops best on cooler mountain ranges, but Liberian and Robusta coffee have a good yield also in the hot-humid lowlands. For optimal cultivation a nutrient-rich soil, as well as favorable water and nutrient supply (N, P, K) are indispensible.

A shrub plant yields well for more than 20 years. The ripe and red fruits are individually hand-picked and the fallen but still intact fruits are collected from the ground. They can remain in the soil for several weeks without loss of quality (NOWAK and SCHULZ 2002). In Brazil, the harvest is carried out in May and September, in Central America in October and April, and in Africa it takes place between March and September (MĂNDIȚĂ 2008).

The products are treated by two methods. In the wet process, the crushed mesocarp is fermented for 24-26 hours following by washing and drying. After this the endocarp and the silverskin are removed mechanically. This method can be used only in the case of ripe fruits. The fermentation affects the quality of the seeds, the external morphological markers and its taste positively. This technique is used mainly in Central America, where the produced coffee is called "milds". In the dry method the fruits are dried in open air, usually they are dried on concrete tables spread out over a period of two or three weeks. They are collected into bunches and covered at night due to the fog. When the pericarp has dried properly, fruits are taken into fermenting warehouses and they are mechanically separated from the mesocarp and silverskin. This procedure is the most popular in Brazil (MĂNDIȚĂ 2008). Finally, the seeds are roasted at 200-250 °C degrees, and the process takes place in the customer country.

The characteristic aroma of the seeds appear as a result of roasting (TÓTH 2010). Coffee oil carries the majority of coffee aroma. A complex mixture of volatile compounds determine the aroma, whereas non-volatiles ones are responsible for the sourness, bitterness, and astringency. The roasting process can be roughly divided into three phases: an initial drying and endothermic phase (the smell of the beans changes from green peasy to bread-like, and the colour turns yellowish), the actual roasting phase (where complex pyrrolytic reactions are characteristic, and the beans become to a dark brown in exothermic and endothermic phases), and a final rapid cooling phase to stop the final exothermic part of the roasting operation using air or water as cooling agents (BUFFO and FREIRE 2004).

The objective of a scientific work was to compare traditional Brazilian Arabic coffee cultivars (Bourbon, Catuaí, and Icatu) with modern cultivars (IAPAR 59, IPR 98, IPR 99, and IPR 103) which were produced under same edapho-climatic conditions. Differences were observed mainly in the content of 5-caffeoylquinic acid, cafestol, and kahweol. In addition, the Arabic cultivars with the introgression of *C. canephora* genes showed high antioxidant activity, and they differed from plants of traditional Arabic genotypes mainly in their diterpene profile (KITZBERGER et al. 2014).

In another work, seasonal changes were monitored in the vegetative growth, leaf gas exchange, carbon isotope discrimination, and carbohydrate status in de-fruited *C. arabica* grown in field from October 1998 through September 1999 in Vicosa, southeastern Brazil. The rate of net carbon assimilation in active growth period was higher than during the period of reduced growth. In the active period, growth, unlike net carbon assimilation or carbon isotope discrimination correlated strongly negatively with air temperature. In contrast, both growth and net carbon assimilation correlated positively, and carbon isotope discrimination correlated negatively with air temperature during the reduced growth period. Change in net carbon assimilation seems to be largely due to stomatal limitations in the active growing season, and with non-stomatal ones prevailing in the slow growth period. Foliar carbohydrates seemed not to have contributed appreciably to changes in growth rates and photosynthesis (SILVA et al. 2004).

The most well-known substitute of coffee is a chicory version extracted from the root of *Cichorium intybus* L. ssp. *sativa* (Lam. et DC.) Janch. convar. *radicosum*. In addition, the fruit of *Ficus carica* L., *Hordeum vulgare* L., *Phaseolus vulgaris* L., *Fagus sylvatica* L. and *Ceratonia*

siliqua L., the root of *Taraxacum officinale* agg., as well as crops and malt can also be used for coffee substitution (HALMAI and NOVÁK 1963, RÁPÓTI and ROMVÁRY 1990).

2.9. Pests

In warm and humid climates, *Coffee* species are susceptible to various infections caused by fungi which kill them and cause destroyed areas of the plants (HINDORF and OMONDI 2011). The most common fungal disease is caused by *Hemileia vastatrix* Berk. & Broome which belongs to Basidiomycota. This disease causes a decoloration with 2 mm yellow spots on the lower surface of the leaves, which shed off spores over time (PATAY et al. 2016c). The hyphae arrive to the middle part of the leaf (mesophyll) through the stomata. After this the young branches fall down, and the internal methabolic system destroyed. The pathogen was discovered in the region of Lake Victoria, Africa in 1861 and it spread to the West Coast as well 20 years later. It was also described in India in 1840, where its appearance resulted total destructions of coffee plantations a few decades later. In Sri Lanka, which was considered a major coffee-exporting country, the pathogen was observed in 1869 caused a decrease in coffee production from 42,000 to 30,000 tonnes within a few years. The wild *C. benghalensis*, *C. lebruniana* and *C. wightiana* were also infected by this fungi in Asia. The disease was discovered in Brazil in 1970, and in Sao Paulo and Parana in 1971. After this Rio de Janeiro made a 50 km wide and 100 km long "quarantine zone" in order to stop the infection. In 1974 the infection appeared at the Paraguay border as well. According to other studies, the hybrid of *C. arabica* and *C. robusta*, which comes from the island of Timor (→ Timor's hybrid), as well as some taxa from San Salvador can be resistant to this disease (BRÜCHER 1977). In comparison with healthy leaves, those infected with *H. vastatrix* displayed an increase in salicilic acid level 24 h after inoculation, which suggests the involvement of an salicilic acid-dependent pathway in resistance reaction of coffees (DE SÁ et al. 2014).

Among other fungi, *Colletotrichum kahawae* J.M. Waller & Bridge, *C. coffeanum* Noack, and *Glomerella cingulata* Spauld. & H. Schrenk, which change the fruits to brown and dry, are responsible for the appearance of coffee fruit-disease ("coffee berry disease"). *G. cingulata* was first described in Kenya in 1920, from where it spread to Central and East Africa. *Cercospora coffeicola* Berk. & Cooke causes a brownish red decoloration at the edge of the leaves. *Rhizochtonia* species spread mainly in East Asia and later in South America, which attack *C. excelsa* and *C. robusta*. *Mycena cirticolor* (Berk. & M.A. Curtis) Sacc, which causes the falling of

coffee leaves, was recorded in Central America. *Gibberella xylarioides* R. Heim & Saccas was observed in Central Africa causing wilting of plants. It mainly affects *C. excelsa* and *C. dewevrei*, but the resistance of *C. robusta* was proved to this infection (BRÜCHER 1977). This fungus can also cause serious problems for producers (HINDORF and OMONDI 2011).

Scientists compared epiphytic and endophytic fungal communities associated with leaves of *C. arabica* in Puerto Rico. A total of 821 colonies were isolated and grouped into 131 morphospecies. The taxonomic affinities of the four most common nonsporulating fungi were determined by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region: two grouped with *Xylaria* and one each with *Botryosphaeria* and *Guignardia*. *Pestalotia* and *Botryosphaeria* were described as significant epiphytes, while *Colletotrichum, Xylaria,* and *Guignardia* as common endophytes. Surprisingly, more morphospecies occurred as endophytes than as epiphytes. Differences in number of fungi per plant were significant among the affected sites; epiphytic and endophytic communities differed on a single leaf, and despite living only millimeters apart, both communities differed from site to site. Significant correlations between occurrence of fungal morphospecies suggested that fungi may have positive or negative effects on their neighbors. This is the first quantitative comparison of epiphytic and endophytic fungi in plants, and the first study of these fungi in coffee leaves (SANTAMARIA and BAYMAN 2005).

"Crespera", a bacteria caused infectious disease of coffees affects both coffee production and the economy of coffee producing countries. This disease causes long and narrow leaves, wavy borders, marginal necrosis, and strong chlorosis during the drying of the leaves. The internodes of the infected plants are short developing multiple sprouts in the axial sprout. The flowers became greenish, the branches possess severe symptoms without apparent external markers and signs. This infection induces strikingly diminishing in coffee bean production (VARGAS et al. 2002).

Meloidogyne exigua is a root-knot nematode parasite, which is considered one of the main responsible factors for reduced productivity in *C. arabica* cultivars in Brazil. The resistance of coffee plants to this parasite is conferred by the gene *Mex-1* (SILVA et al. 2013).

The first description of red mite appearance (*Oligonychus ilicis* McGrgor) was found in 1950 in coffee species. The red mite lives on the upper surface of coffee leaves, on which the females spin delicate webs and lay their eggs. The main external symptom of the infestation is the loss of characteristic shine of the leaves by becoming bronze-coloured (FAHL et al. 2007). An association between increased egg laying by the leaf miner and increased caffeine levels in coffee leaves was

recently recognized. However, since caffeine is not volatile, its effect on egg laying is likely aided by a volatile compound. The recognized active volatiles were subjected to partial correlation against the density of eggs laid by the leaf miner in each coffee genotype. Among volatile components, *p*-cymene was subjected to an attraction test in a four-arm olfactometer confirming its biological activity and providing evidence of its role stimulating egg laying by coffee leaf miner (MAGALHAES et al. 2008).

The Class III Chitinase PR-8, signal peptidase complex subunit SPC25, photosystem gene psaH, a putative calcium exchanger similar to CAX9, and a homeotic gene BEL may have a possible function in coffee resistance, coffee development and a hypothetical defense mechanism against *Leucoptera coffeela* Guérin-Méneville (MONDEGO et al. 2005). Mechanical damage, Meja treatment, *H. vastatrix* fungus inoculation and *Leucoptera coffeella* infestation cause different responses in polyphenoloxidase activity, which suggest that coffee resistance may be related to the oxidative potential of the tissue regarding the phenolic composition (MELO et al. 2006).

The coffee berry borer (*Hypothenemus hampei* Ferrari) was accidentally introduced to Brazil. The fertilized female bores the fruit in the disc region making a gallery through the pulp. Than it bores the seeds (grains), and lays the eggs inside. After hatching, larvae feed on the seeds by destroying them totally or partially. The reproduction of this insect is higher in highly humid environments. Investigations showed that *C. kapakata, P. benghalensis, C. eugenioides,* and genotypes with *C. eugenioides* genes were resistant to this pest. *C. eugenioides* and *C. kapakata* could present resistance only at epicarp level, while *P. benghalensis* in the seeds as well (SERA et al. 2010).

The role of peroxidase was determined in coffee stems during invasion by *Cuscuta jalapensis* (parasitic plant). The results showed that the activity of peroxidase is associated with the processes of invasion and the destruction of tissues of the host up until the moment when the haustorium makes contact with the vascular bundles of the affected plant. The peroxidase activity in the cell walls of *Cuscuta jalapensis* is strongly associated with the morphogenesis of connecting structures, as well as with the processes leading to invasion of host plants (LOPEZ et al. 2006a).

2.10. Phytochemical analyses of plant parts of coffees

In addition to the abovementioned groups of detected compounds of coffee taxa, several studies aimed their identification according to the plant parts which are shortly reviewed in the following separated section.

2.10.1. Coffee seed

Nowadays there are about 8000 known phenolic compounds with aromatic ring produced as secondary metabolites in plants. They protect plants against pathogens and abiotic stress such as change of temperature, water content, exposure to UV light, and deficiency of mineral nutrients (MONTEIRO et al. 2012). They contain two carbon frameworks namely hydroxycinnamic and hydroxybenzoic structures. Caffeic, *p*-coumaric, vanillic, ferulic, and protocatechuic acids are present in nearly all coffee taxa (STALIKAS 2007).

In *Coffea* species, 5-caffeoylquinic acid (5-CQA) is the most abundant soluble ester. The beans of *C. canephora* contain feruloylquinic acids (3-, 4- and 5-FQA), and the isomers of monoester (3-, 4- and 5-CQA) and diester (3,4-, 3,5- and 4,5-diCQA) CQAs. Hydroxycinnamoylquinic acids are involved in the bitterness of coffee beverage due to their degradation into phenolics during roasting (MONDOLOT et al. 2006). In addition, iridoid glycosides, tannins, and anthraquinones have also been detected in coffees (WIART 2006).

The official drug is the seed (*Coffeae semen*) containing 1.25-2.5% caffeine (roasted seeds: 1.36-2.85%), theobromine, theophylline, 4.4-7.5% chlorogenic acid (roasted seeds: 0.3-0.6%), 0.8-1.25% trigonelline (roasted seeds: 0.3-0.6%), 0.022% choline, 10-16% fatty oil, quinic acid, sitosterol, dihydrositosterine, stigmasterol, coffeasterin, tannin, wax, caffeic acid, sugar, cellulose, hemicellulose, non-volatile aliphatic acids (citric, malic, and oxalic acid), volatile acids (acetic, propanoic, butanoic, isovaleric, hexanoic, and decanoic acids), soluble carbohydrates (e.g. monosaccharides: fructose, glucose, galactose, and arabinose), oligosaccharides: sucrose, raffinose, and stachyose, ad well as polymers of galactose, mannose, arabinose, and glucose (BUFFO and FREIRE 2004, KRAFT and HOBBS 2004, ESQUIVEL and JIMÉNEZ 2012). The concentration of caffeine, which occurs partially in free form or as salt with chlorogenic acid, is reduced during roasting (FATTORUSSO and SCAFATI 2008). Theophylline is used as an important smooth muscle relaxant in bronchospasms in combination with ethylenediamine (aminophylline)

or choline. These alkaloids are either extracted from natural sources or produced by partial or total syntheses (DEWICK 2002).

Coffee seeds are rich in biologically active substances and polyphenols such as kaempherol, quercetin, ferulic, sinapic, nicotinic, quinolic, tannic, and pyrogallic acids which possess antioxidant, hepatoprotective, antibacterial, antiviral, anti-inflammatory, and hypolipidaemic effects (HUANG et al. 1988, MUSSATTO et al. 2011, NARAYANA et al. 2001, YEH and YEN 2003, NAIDU et al. 2008, BREZOVA et al. 2009, DZIKI et al. 2014, GÎRD et al. 2014, QUIROZ et al. 2014). Besides the *cis*-isomers of chlorogenic acid in Arabic coffee (CLIFFORD et al. 2008), high quantity of esters of feruloylquinic acid was described in the fruit and the green bean of a wild species namely *C. pseudozanguebariae* (CAMPA et al. 2012). The Arabic and Robusta coffee seeds contain 10-16% fatty oil in droplets as a storage substance (SZŐKE and KÉRY 2003, SIMKIN et al. 2006). The oil of the immature seeds can be used as an alternative source of biodiesel, and the seed coat can be applied for bioethanol production. Seed coat and cakes pressed from defective beans are useful as adsorbents for the removal of dyes from aqueous media (COSTA et al. 2014). The lipid fraction of the seeds including waxes, oils, and unsaponifiable materials plays an important role in the embryonic development (WAGEMAKER et al. 2011)

The biochemical studies of wild species from Africa and Madagascar focused on qualitative and quantitative assessments of sugar, lipid, caffeine, and esters of hydroxycinnamic acid (HCE) in green coffee beans. However, there are many studies on beans, relatively few experiments deal with the metabolite content of other plant parts (e.g. leaf, outer fleshy layer of the fruit, etc.). Moreover, many of these studies are still focusing on cultivated species investigating mostly their HCE content (CAMPA et al. 2012).

A cup of coffee contains about 1 mg of vitamin PP (nicotinic acid). Three cups of coffee, which is the maximum suggested quantity of coffee per day, provides 25 to 50% of the daily requirement of this vitamin (MĂNDIȚĂ 2008).

The whole or grounded silverskin can be used as a possible fertilizer after compost-production as a versatile barrier in reduction of pesticide which leach through the soil, and as a source of oil for biodiesel or biomass for bunker fuel substitution (COSTA et al. 2014). The wasted coffee contains carbon (>58%), nitrogen (<2%), ash (<1%), lipophilic fractions, ethanol, water-soluble compounds, components solubilized in 1% NaOH, lignin, polysaccharides, glucose, mannose, free

fatty acids like *n*-hexadecanoic acid, polyphenols, tannins, lipids, polysaccharides, and chlorogenic acid (PUJOL et al. 2013).

The concentration of trigonelline and sucrose (aroma precursors of coffee species) was also evaluated in *Coffea* species. The trigonellin content varied between 0.39% and 1.77%, while the sucrose content changed from 3.8% to 10.7% of dry plant material of 20 selected species. The highest concentration was measured in the green beans of *C. arabica*, *C. kapakata* and *C. salvatrix* (CAMPA et al. 2004), while *C. canephora* contained more chlorogenic acid and caffeine (KY et al. 2001, CAMPA et al. 2004)

The characteristic aroma of coffee is due to α-2-furfurylthiol, 4-vinylguaiacol, some alkyl tyrosine derivatives, furanones, acetaldehyde, propanal, methylpropanal, and 2- and 3-methylbutane content (BUFFO and FREIRE 2004, KRAFT and HOBBS 2004). Cafesterol and bengalensol have also been isolated and identified by various chromatographic techniques in *C. benghalensis* (ASHIHARA and CROZIER 1999, BEGUM et al. 2003, KAISAR et al. 2011).

Carotenoids, which are generally present in leaf, flower, fruit, and shoot of plants, play an important role in the stabilization of lipid membranes, photosynthesis, and protecion against strong radiation and photooxidative processes. Experiments with coffee species also showed that the transcript levels of enzymes involved in the synthesis of carotenoids increased under stress conditions (SIMKIN et al. 2008).

Coffee taxa contain inorganic materials like K, Mg, Ca, Na, Fe, Cu, Mn, Zn, Rb, Sr, V, Co, Ni, Ba, B, minerals, and a minor amount of B, C, P, and PP vitamins (PÂRVU 2000).

2.10.2. Coffee leaf

Some studies aimed at the comparison of these compounds among various *Coffea* species and their plant parts. The total phenolic content of young leaves was not influenced by fruit production, but it strongly depends on the developmental stage of plants and the environmental factors, which correlated inversely with the temperature and radiation (SALGADO et al. 2008).

Campa et al. (2012) studied the presence of mangiferin and hydroxycinnamic acid esters in the leaf of 23 African coffee species. They found that the total hydroxycinnamic acid content of *C. arabica* was significantly higher than that of other species (e.g. *C. sessiliflora*, *C. resinosa* and *C. leroyi*).

Mangiferin and isomangiferin were present in higher concentration in the young leaves than in other plant parts (CAMPA et al. 2012, TALAMOND et al. 2008). 3,4-dicaffeoylquinic acid was detected in *C. canephora*. Feruloylquinic acids were present in higher amount in *C. stenophylla* than in *C. arabica* and *C. humilis*. The caffeoylquinic acid content of the adult leaves of *C. canephora* was 10 times lower compared to the young ones (MONDOLOT et al. 2006). *C anthonyi* and *C. salvatrix* presented higher concentration of mangiferin than *C. arabica, C. eugenoides, C. heterocalyx, C. pseudozanguebariae,* and *C. sessiliflora* (CAMPA et al. 2012, FARAH and DONANGELO 2006).

Based on our investigations, phenolic acids such as caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic acids, as well as rutin, quercetin, kaempferol, and isoquercitrine were indentified in the leaf of Arabic and Bengal coffee (PATAY et al. 2013). *Cis*-isomers of chlorogenic acid were observed in higher amount in the leaf than in the seeds of Arabic coffee. This fact may suggest that UV radiation can provoke geometric isomerisation of chlorogenic acid in leaves (CLIFFORD et al. 2008). Recent researches have great emphasis on the physiological effect of the infusion of *C. arabica* and *C. canephora*, and their mangiferin and isomangiferin content. Mangiferin was initially isolated from the leaves, bark, and peel of mango *(Mangifera indica* L.), and it is well-known for its numerous pharmacological properties such as anti-inflammatory, antidiabetic, antihyperlipidaemic, and neuroprotective activities, as well as it provides antioxidant and antimicrobial effect in biotic stress (CAMPA et al. 2012). Mangiferin and isomangiferin were identified in the leaves of *C. pseudozanguebariae,* and in other species of Rubiaceae family used MS and NMR techniques (TALAMOND et al. 2008, CAMPA et al. 2012). In addition to *C*-glucosylxanthone and mangiferin, high quantity of feruloylquinic acid esters was firstly detected in the leaf of *C. pseudozanguebariae*. Dicaffeoylquinic acids, 5-CQA, and feruloylquinic acids were also detected in the leaf of *C. canephora* (MONDOLOT et al. 2006).

The presence of monoterpenoid alkaloids is characteristic to Rubiaceae family. Several enzymes take part in the synthesis of purine alkaloids such as caffeine synthase, xanthosine 7-*N*-methyltransferase, 7-methylxanthine 3-*N*-methyltransferase, caffeine xanthine methyltransferase 1 (CaMXMT1), caffeine methylxanthine methyltransferase 2 (CaMXMT2), caffeine dimethylxanthine methyltransferase (CaDXMT1), and theobromine 1-*N*-methyltransferase (ANISZEWSKI 2007).

An adenosine nucleosidase was purified from the young leaves of *C. arabica* cv. *Catimor* showing maximum activity at pH=6.0 in citrate-phosphate buffer. It hydrolyses adenosine to adenine and ribose in the purine metabolic pathway, which enables the recycling of these metabolites. It seems to be important in catabolizing tissues e.g. in cotyledons, which makes adenine available for transport and reutilization in other organs. In addition, it has a possible role in the inter-conversion of these growth regulators (interchange between cytokinin base, riboside, and ribotide forms), and consequently in cytokinin transport and activity regulation in plants (CAMPOS et al. 2005). Polyphenol oxidases are active mostly in the early developmental stages of the leaf and the endosperm e.g. in *C. arabica* (MAZZAFERA and ROBINSON 2000).

Caffeine, the most important alkaloid of coffee species, is synthesized in the young leaves of e.g. *C. arabica* seedlings and immature fruits, and it is accumulated in the mature leaves. The active caffeine biosynthesis is carried out in the upper leaves and the upper part of the stem, but it is absent in the second and third leaves, cotyledons, lower stem, and root (ASHIHARA et al. 2008).

C. liberica is less cultivated due to its lower caffeine content (1.8% dry matter basis), and to its sensitivity to *Fusarium xyloriodes* (N'DIAYE et al 2007). The leaves of *C. salvatrix*, *C eugenioides* and *C. benghalensis* contain 3-7 times less caffeine than *C. arabica*, hence they are rarely studied and used in the industry nowadays. The degradation of caffeine, which is negligible in the leaves of Arabic coffee, is also very slow in *C. salvatrix* and *C. benghalensis*, but its catabolism is faster in the young and mature leaves of *C. eugenioides*. Catabolism patways involve the conversion of caffeine into theophylline, 3-methylxanthine, xanthine, uric acid, allantoin, allantoic acid, urea, and finally it results in CO_2 and NH_3 (ASHIHARA and CROZIER 1999).

2.10.3. Coffee coal

Coffee coal (*Coffeae carbo*) is obtainable by the charring of roasted coffee beans. It contains caffeine (75% of the original amount), phenols, tannins, trigonelline, chlorogenic and caffeic acids. The IVth Hungarian Pharmacopoeia used coffee syrup (*Syrupus coffeae*) as a flavor corrigent which was later substituted with chicory syrup (*Syrupus cichorii*). During roasting, coffee seed swells and turnes brown, and it partially loses its water content. The sugar is caramelised, then aromatic substances are produced, which provide the characteristic aroma of coffee beverage (HALMAI and NOVÁK 1963, RÁPÓTI and ROMVÁRY 1990). Generally, more than 90% of α- and β-tocopherols remain unchanged after roasting except Robusta coffee, in which the concentration of

β-tocopherol is reduced by 25% after roasting. This change was not observed in Arabic coffee (ALVES et al. 2009).

2.11. Histological localisation of metabolites

The occurrence of chlorogenic acid and mangiferin in *C. pseudozanguebariae*, *C. eugenioides*, *C. arabica* and *C. canephora* was studied by spectral analysis combined with advanced linear unmixing. Based on the differences in phenolics of young leaves, the aforementioned 4 species were classified into two groups. In the first group (*C. p.* and *C. e.*), based on the presence of 5-CQA and mangiferin, histochemical difference was detected between adaxial and abaxial epidermal tissues: 5-CQA fluorescence was observed in vascular bundles and in the cuticle of the abaxial epidermis, and lesser in the cells of the adaxial epidermis (it was not detected on the adaxial cuticle). Mangiferin fluorescence was detected in cuticles and in all parenchyma cells (like spongy cells) and at the lower level of the palisade parenchyma. In the second group (*C. a.* and *C. c.*), mangiferin was not present in the epidermis, only in the parenchyma (as small vesicles), but 5-CQA could be identified in the vacuoles of both tissues (CONÉJÉRO et al 2014).

Based on histochemical and microspectrofluorometrical analysis, caffeoylquinic acids (mono- and di-esters) were closely associated with the chloroplasts in young leaves of *C. canephora*. During leaf ageing, they intensively accumulate in chlorenchymatous bundle sheath cells and then in the sclerenchyma cells around the phloem. The association with chloroplasts suggests a protective role of caffeoylquinic acids against light damage. In older leaf tissues, they are transported through the phloem which confirms their role in lignification. Similarly to other phenolics, hydroxycinnamoylquinic acids are accumulated in vacuoles or in apoplast. Their biosynthesis apparently takes place with enzymes in the chloroplast. In *C. canephora*, hydroxycinnamoylquinic acids accumulate in beans, where their content can exceed 10% of dry bean weight. Because it is able to form complexes with caffeine, it is thought to participate in the vacuolar sequestration of this alkaloid. Insoluble phenolics are distributed in cell walls, while soluble compounds are compartmentalised within cell vacuoles. Feruloylquinic acids (3-, 4- and 5-FQA) and caffeoylquinic acids, i.e. the isomers of monoester CQA (3-, 4-, and 5-CQA) and diesters can also be found in the green beans of the species (MONDOLOT et al. 2006).

2.12. Adaptation to inadequate environmental conditions

Thermal analyses of seed tissues of *C. liberica* suggested that the absence of freezable water is an important factor for successful cryopreservation of excised coffee embryos; their optimal desiccation was carried out at 17.17% moisture, 30 min (HOR et al. 1993). Some cultivars of *C. arabica* presented similar tolerance for desiccation and low temperature. Based on earlier reports, *C. racemosa* was the most tolerant while *C. liberica* the least tolerant for desiccation (MIRIAN et al. 1999). Cold conditions have a severe impact on the growth, development, photosynthesis, and production of coffees. Studies related to the acclimation ability of *C. canephora* cv. *Apoata*, *C. arabica* cv. *Catuaı´*, *C. dewevrei*, and two hybrids namely *Icatu* (*C. arabica* x *C. canephora*) and *Piata* (*C. dewevrei* x *C. arabica*) showed, that *Catuaı´* had an intermediate response through the reinforcement of some antioxidative molecules, usually to a lesser extent than that of *Icatu*. *C. dewevrei* showed the poorest response in terms of antioxidant accumulation, and also showed the greatest increase in OH values (FORTUNATO et al. 2010).

2.13. Ethnobotanical data

The recorded ethnobotanical data are reviewed according to the used parts of coffees as follows.

The beverage prepared from the **seeds** of Arabic coffe was used to treat flu in Brazil, whereas the hot water extract of the seeds was known as an aphrodisiac drug in Cuba. In Haiti, the infusion of the roasted seeds and the leaves were used for anemia, edema, and asthenia. The decoction of the seeds was used orally for fever and as an astringent drug in Nicaragua (ROSS 2005), while the aqueous extract of the dried seeds are known for tiredness in Peru, as a cardiotonic and neurotonic drug in Thailand, and for asthma in West India (ROSS 2005).

Dried **fruits** boiled in water are known as a beverage called "giser" in Yemen (NOWAK and SCHULZ 2002). The seeds of *C. canephora* are used locally as a massage for backache (TABUTI et al. 2003). In Ethiopia, native people consumed a beverage called "hoja" to treat poisoning which is accompanied by diarrhea and nausea. This beverage made of the pericarp of coffee berries contained milk and honey (BELAYNEH and BUSSA 2014). In Africa, coffee seeds are used against asthma attacks (NEUWINGER 2000).

The **leaf** sap of Arabic coffee was consumed to treat diarrhea and intestinal pain in Africa. It is used to manage HIV/AIDS in Kamuli, Sembabule, Kabale, and Gulu districts of Uganda (LAMORDE et al. 2010). In Cuba, people used the leaves both orally or locally to treat migraine;

sometimes they use the leaves in fresh form or as an infusion placed onto the painful body parts. Coffee leaves are also mentioned for headache and stomach pains as a decoction in Nicaragua, as cough suppressant as an infusion in Peru, as well as for fever and stimulation of prolactin's production in Mexico (ROSS 2005). In Indonesia and Ethiopia, people name the tea of the leaf of Arabic or Robusta coffee "copi daon" or "leaf coffee". In Liberia, the infusion of the leaf of Arabic coffee was consumed only for its taste, but when it was tried to be sold at the markets of London, it was not admitted to be popular, rather undrinkable (CRAMER 1957). In Africa, the tea prepared from the leaf of Robusta coffee was used for bleeding accompanying abortion: chopped leaves were mixed with 0.5 L of water, then it was squeezed and consumed (NEUWINGER 2000). Dried leaves are still used for preparation of a tea named "Quti" in Ethiopia (GIDAY et al. 2010). The salty drink (tea) of the leaf of Liberian coffee was described as a laxative drug (BELAYNEH and BUSSA 2014). In the case of *C. canephora,* leaves were documented for cough and jaundice orally as a decoction and infusion (TABUTI et al. 2003).

The **flowers** of *C. benghalensis* were known for excessive bleeding during menstruation in Nepal (GHIMIRE and BASTAKOTI 2009).

The **root** sap of *C. arabica* was consumed for scorpion bites in West India (ROSS 2005) and it was chewed or added to food to obtain an aphrodisiac effect in Ethiopia (BELAYNEH and BUSSA 2014). The root extract of *C. canephora* was described for measles as a bandage for children (TABUTI et al. 2003).

In folk medicine, the coffee **coal** (charcoal) of *C. arabica* was documented to use for purulent wounds, pharyngitis, and stomatitis (GRUENWALD et al. 2000).

2.14. Medicinal importance and application of coffees

Coffee has a well-known stimulant effect on the central nervous system (KOTHE 2008). It can increase the effect of painkillers (NEUWINGER 2000, KHARE 2007, MILLS et al. 2006) or reduce tiredness (KHARE 2007). American researchers have shown that the regular consumption of coffee and cola may reduce the incidence of Parkinson's disease (STÁJER 2004). In addition, coffee can be used in migraine therapy combined with ergotamine, because caffeine has a vasoconstrictor activity in the brain (STÁJER 2004).

In oxidative stress, reactive oxygen species (ROS) have been suggested to participate in the initiation and propagation of chronic diseases such as cardiovascular and inflammatory diseases,

cancer, or diabetes (DURAK et al. 2014). Antioxidants, which are found naturally in many foods and beverages, provide health benefits in the prevention of heart diseases and cancer by fighting cellular damage caused by free radicals in the body. In general, consumers prefer natural antioxidants because they are considered to be safe and environmental friendly (RAMALAKSHMI et al. 2008).

The antioxidant activities of the seed extracts of *C. arabica* were measured by ferric reducing power assay for water-soluble components using ascorbic acid, and by Rancimat assay for lipid-soluble portions using sunflower oil as a substrate. All coffee extracts improved the oxidative stability of sunflower oil. Based on earlier study on the caffeine content of various African coffee species by Gebeyehu and Bikila, *C. arabica* originated from *Wembera* region has higher caffeine content and higher antioxidant activity than *C. arabica* from *Burie, Goncha,* and *Zegie* in the continent (GEBEYEHU and BIKILA 2015). The caffeine content of the silverskin of *C. arabica* was found to be 3.5 times lower than in most coffee brews. The total antioxidant capacity of this part was similar to those of valuable sources of food antioxidants like dark chocolate, herbs, and spices (BRESCIANI et al. 2014). The extracts of the silverskin of Arabic and Robusta species prepared by supercritical fluid extraction showed antioxidant activity used DPPH radical scavenging capacity and H-ORAC antioxidant assays (NARITA and INOUYE 2012).

Coffee seed extract may have an important role in UV protection and cancer prevention because of its antioxidants. In a clinical study, 30 patients suffered from dermatological problems were treated locally with seed extracts: the tested cream was applied to the whole facial area in 20 patients and only to the half of the face in 10 patients, where the remaining area was treated with a placebo cream. Compared with placebo, the investigated product improved visibly the appearance of fine lines and reduced wrinkles and pigmentation, but above all it improved the formation of the facial skin (COOPER and KRONENBERG 2009).

In another study, the antioxidant activity and the inhibitory effect of skin tumor promotion of ferulic, chlorogenic and caffeic acids were determined in mices. They were applied to the skin of mices together with 5 nmol of 12-O-tetradecanoylphorbol-13-acetate (TPA). As a result, they inhibited the number of TPA-induced tumors by 60, 28 and 35%, respectively in each mouse, in addition, the increasing concentration of phenolic acids caused more pronounced inhibitory effect (HUANG et al. 1988). Sinapic, caffeic, and p-coumaric acids also showed antioxidant capacity and modifying effect on sulfate conjugation (YEH and YEN 2003). The β-cyclodextrin-enclosed

chlorogenic acid showed about 2 times higher antioxidant capacity than the crude and purified extract (BUDRYN et al. 2014).

However, *C. liberica* is less used, the volatile extract of its immature beans possessed higher antioxidant capacity than that of *C. arabica* and *C. robusta* (CHEE et al. 2015). The fruit of Bengal coffee showed stronger antioxidant activity than ascorbic acid and α-tocopherol (KIRAN et al. 2011).

The regular consumption of coffee reduces the feasibility of cancers of kidney, liver, premenopausal breast, and colon, which is also due to caffeine, diterpenoid, caffeic acid, polyphenol and essential oil content, and heterocyclic molecules. However, no correlation was found between coffee intake and prostate, pancreatic, and ovarian cancers nowadays (NKONDJOCK 2009). The anticancer effect of the fruit extracts of *C. arabica* has also been confirmed in mouse studies (ROSS 2005).

A cup of coffee contains about 80 mg of caffeine, but e.g. the high quality Hungarian short coffee contains only approximately 50 mg of caffeine, exactly as much as a cup of tea. Caffeine is rapidly absorbed in the gastrointestinal system bounding to proteins and being transported to many places in the human body. The blood plasma level reaches its maximum 30 min after the intake, and the half-life is five hours. In the metabolic phase, caffeine is dimethylated and degraded to theobromine, theophylline, and paraxantine. Due to caffeine, coffee beverage has a diuretic and a temporarily hypertensive effect, increasing the secretion of stomach acid, as well as it stimulates the function of the heart and the kidney (STÁJER 2004). The theobromine content of the seed also has a diuretic and spasmolytic effect similarly to theophylline (DEWICK 2002).

The aromatic substances increase the gastric secretion (BABULKA et al. 2012): seven cups of coffee per day can increase the gastric acid level and suicidal tendency used simultaneously with alcohol and cigarette (ROSS 2005). The choleretic-cholagogue effect of chlorogenic acid was also documented: in the stomach, chlorogenic acid is hydrolyzed and converted to caffeic acid which conjugated to form the glucuronate or sulphate (WILLIAMSON et al. 2009). Chlorogenic acid inhibits the glucose-6-phosphatase enzyme which can decrease the glucose production in the liver, while caffeine stimulates the insulin production of β-cells in the pancreas (KHARE 2007).

Recently, some clinical studies confirmed that coffee consumption is not as harmful as one might think. According to an epidemiological study, the active substances of coffee seeds can also reduce the disorders of the gallbladder (BABULKA et al. 2012). Regular coffee consumption can reduce the

probability of type II diabetes by 60% in the case both of caffeinated and decaffeinated beverages, therefore caffeine is not involved in this effect, but it is still unknown which substances can be responsible for this (BISHT and SISODIA 2010, BELGUIDOUM et al. 2014).

Coffee has hepatoprotective effect also regardless of the decaffeination process. Roasted coffee brews have a stronger protective effect against liver damage than green coffee brews (LIMA et al. 2013). The stomach irritating tannins, which are present in the beverages of coffee and tea shrub (*Camellia sinensis* L.), can be neutralised by milk (PETRI 2006). Coffee drinks can also reduce the absorbtion of nicotine of nicotine-containing chewing gums (WILLIAMSON et al. 2009).

The powdered leaf and stem of *C. arabica* can provoke allergic reactions. Scientific tests have proven the anti-inflammatory activity of locally applied green seed extracts (ROSS 2005). Kaempferol, rutoside and quercetin showed an antiulcer activity in rats (NARAYANA et al. 2001), and an antiviral, antifungal, antibacterial, anticellulitic, anti-aging (BOROS et al. 2010, QUIROZ et al. 2002), anti-inflammatory and antiallergic activity, as well (NARAYANA et al. 2001). Coffee coal possesses astringent effect (BABULKA et al. 2012), while *in vitro* and *in vivo* studies showed that the hydroalcoholic extracts of the silverskin has no irritating effect on the skin and it can be used in cosmetics (RODRIGUES et al. 2015).

Linolenic acid, which is present in high concentration in green coffee seeds, blocks the harmful UV-rays and it has a sunscreen effect, which property is useful in cosmetics. In another study, the concentration of seed waxes, oils, unsaponifiable materials, and the sunscreen effect were analysed in 10 coffee species: linoleic acid was found in high concentration, wax from 0.0 to 2.8%, oleic acid from 6.9 to 32.4%, and the unsaponifiable matters from 0.3 to 13.5%. The sunscreen effect was between 0.0 and 4.1 SPF depending on the tested coffee species (WAGEMAKER et al. 2011).

Isoquercitrin and rutoside extracted from coffee seeds can be used for atherosclerosis, while quercitrin has a positive chronotropic, positive inotropic, and antiarrhythmic effect, as well as it protected LDL against oxidative modifications in guinea pig. Quercetin and rutoside have been used in the treatment of capillary fragility and phlebosclerosis (NARAYANA et al. 2001).

Coffee seeds can also decrease the blood sugar level (ROSS 2005). In the therapy, caffeine can be used for patients with mild cardiovascular diseases to increase blood pressure, body temperature, and blood circulation (STÁJER 2004). The single ingestion of coffee polyphenols improved the peripheral endothelial function after glucose loading in healthy subjects (OCHIAI et al. 2014). The

aqueous extract of *C. arabica* possessed *in-vitro* anti-inflammatory effect against protein denaturation on egg albumins which is also due to the polyphenol content (CHANDRA et al. 2012).
Coffee seeds can be used as a respiratory stimulant, in addition, an unexpected effect of caffeine is to promote quitting smoking (STÁJER 2004).
Some studies have shown that coffee consumption can cause spontaneous abortion during pregnancy due to its caffeine content (ROSS 2005). In addition, the use is also not recommended during lactation, because caffeine can appear in the breast milk (ISTUDOR 2005).
Although, some data suggest that caffeine is effective in weight-loss diets through e.g. thermogenesis and fat oxidation (RUDGLEY 2008, WESTERTERP et al. 2005, LOPEZ 2006b, LIU et al. 2013), it is still not fully known. The topical anticellulite effect of siloxanetriol alginate caffeine has also been shown by histological evaluation of fatty tissues in Wistar rats where the diameter of the fatty cells was reduced by 17% (VELASCO et al. 2008).
Recent studies have shown that the fruits of Bengal coffee have antibacterial properties against *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumonia*, *Vibrio cholerae*, *Salmonella typhimurium*, *Salmonella typhi*, *Streptococcus faecalis*, and *S. aureus* (KIRAN et al. 2011, SHETTY et al. 1994, ALMEIDA et al. 2006). Due to chlorogenic acid and Maillard reaction products which are generated during seed roasting, the extract of *C. canephora* showed an inhibitory effect against *Streptococcus mutans* which can cause tooth decay (ANTONIO et al. 2011a,b, FERRAZZANO et al. 2009, ALMEIDA et al. 2012).
In a veterinary medicinal study, 10 mL of Arabic coffee seed extract were injected under the skin of newborn calves which increased the healing time of the animals by 30% in diarrhea compared with placebo controls. In Swiss provinces like Aargau, Zurich and Schaffhausen, farmers brewed drink of coffee seeds to treat reproductive, gastrointestinal and metabolic disorders, as well as for the infertility of animals (SCHMID et al. 2012).

2.14.1. Adverse effects of coffee

Coffee is consumed mainly as a drink all over the world owing to its stimulating effect, but an overdose of caffeine can cause serious secondary effects (KOTHE 2008), like intestinal irritability, diarrhea, nervousness, stomach ulcers, anxiety, pancreatic cancer, cholesterol, and an abnormal rise. These abnormalities are caused by the use of coffee combined with excessive tobacco and/or alcohol, as well as by the solvent residues used in decaffeinisation (BABULKA et al. 2012). If

caffeine concentration exceeding 1.5 g/day, serious side effects may appear like high blood pressure, numbness, muscle spasms, hallucinations, long term spasm of the back muscles, arrhythmic tachycardia, epileptiform convulsions, and respiratory paralysis (STÁJER 2004, ISTUDOR 2005). Moreover, the excessive coffee consumption (more than 5 cups daily) can cause caffeine addiction namely caffeinism. This overdose can provoke irritability, tremors, sleep disturbances, vomiting, diarrhea, headache (KRAFT and HOBBS 2004), and rarely death, which was recorded mostly in children. First symptoms include vomiting and stomach cramps. The artificial induction of vomiting, gastric lavage, administration of diazepam and spasmolytical substances are used as first aid (STÁJER 2004, ISTUDOR 2005). The less well-known effects of caffeine include drowsiness among the elderly: it increases the cardiac output and improves blood circulation of the brain, and it makes falling asleep easier (STÁJER 2004). The lethal dose for adults extends from 150 to 200 mg caffeine/kg.

Coffee consumption is not recommended for people suffering from high blood pressure, coronary heart disease, kidney and certain neurological disease, stomach ulcer, hyperthyroidism, anxiety, caffeine sensivity, as well as during pregnancy and for children (ISTUDOR 2005, STÁJER 2004).

2.14.2. Interactions

Patients taking regularly medicines may drink coffee carefully because several interactions were described. Excessive coffee consumption can decrease the concentration of vitamin B but it increases the effect of aspirin and paracetamol (KHARE 2007). Human serum albumin also interacts with chlorogenic acid of coffee with high affinity. This statement was studied by fluorescence spectroscopy using eight polyphenol compounds, four acids (caffeic, ferulic, 5-O-caffeoyl quinic, 3,4-dimethoxycinnamic acid), and four lactones (3,4-O-dicaffeoyl-1,5-c-quinide, 3-O-[3,4-(dimethoxy)cinnamoyl]-1,5-c-quinide, 3,4-O-bis[3,4-(dimethoxy)cinnamoyl]-1,5-c-quinide, 1,3,4-O-tris[3,4-(dimethoxy)cinnamoyl]-1,5-c-quinide) extracted from coffee seed. In this study, the dissociation constants of the albumin–chlorogenic acids and albumin–quinides complexes were observed in the micromolar range between 2 and 30 μM (SINISI et al. 2015).

2.14.3. Caffeine-free and decaffeinated products

Caffeine-free species are found at the islands of the Indian Ocean (*C. humbertii* J.-F. Leroy, *C. vatovavyensis* J.-F. Leroy), and in Africa (*C. pseudozanguebariae*, *C. charrieriana* Stoff. & F. Anthony) (HAMON et al. 2015).

Nowadays, many decaffeinated coffee beverages are known prepared by chemical solvents, water, and supercritical fluid extraction. These methods may be harmful to the human body which lead to studies to obtain new and less harmful extractions. The microbiological caffeine metabolisation using *Pseudomonas* and *Aspergillus* strains, its enzymatic pathway, as well as the genetical reduction of caffeine can be mentioned as the basic points of these analyses in plants (GOKULAKRISHNAN et al. 2005). The decaffeinisation of coffee seeds is not completely effective, because decaffeinated coffee also contains a minimum quantity of caffeine (0.08%) (KRAFT and HOBBS 2004).

3. Histological study of the selected *Coffea* species

The histological characters of plants, namely "internal morphological characters" play also an important role in plant taxonomy, which provide a more complex description of plants accompanying the chemotaxonomical field.

Recently, few histological data were published on the leaves and fruits of coffee species nowadays, which focused mostly on Arabic and Robusta coffee. Due to this reason our work aimed to complement these features in the case of Bengal, Liberian and Arabic coffee, and to emphasize the taxonomical differences of cultivated and wild coffee species which were growed at the same conditions.

3.1. Materials and methods

The histo-anatomical investigations were carried out at the Department of Pharmacognosy, Faculty of Pharmacy, and the Institute of Biology, Faculty of Science, University of Pécs.

3.1.1. Chemicals

The following chemicals were used for the analysis: ethanol, acetone and glycerine purchased from Molar Chemicals Ltd., Hungary, as well as distilled water, xylol, paraplast, toluidine blue, Canada balsam, KOH, H_2O_2 and Neo-Mount® dye from Merck Ltd., Hungary.

3.1.2. Sample preparation

The stem, leaf, petiole, and imature fruits of *C. arabica* (Fig. 3), *C. benghalensis* (Fig. 4), and *C. liberica* (Fig. 5) were collected in the Botanical Garden, University of Pécs in the spring of 2014 and 2015. According to the documentation of the garden, *C. arabica* was ordered from D-Konstanz, Germany in 2008 (plantage into the garden: 02/26/2009), *C. liberica* from D-Giessen, Germany in 2011 (plantage: 04/17/2012), while in the case of *C. benghalensis* there are data only about the age of the plant (~10 years old). In summer these plants are outside but in winter in greenhouse at 12-15 °C and 40-60% humidity. Pécs is situated at 193 m altitude above the sea level and at latitude 46°04′59″ N and longitude 18°13′59″ E. Voucher specimens of each species were deposited and labelled with unique codes at the Department of Pharmacognosy, University of Pécs.

Samples were collected from 2 plants per each species: 10 leaves were air-dried at room temperature in shade for leaf clearing method, and 10 other ones were fixed in a mixture of 96% ethanol:glycerine:water (1:1:1) for the histological preparation. Altogether 10-10 stems, petioles and fruits were also fixed in this mixture for preparation of each species.

Fig. 3. *C. arabica* L. (Botanical Garden, University of Pécs)

Fig. 4. *C. benghalensis* (Botanical Garden, University of Pécs)

Fig. 5. *C. liberica* (Botanical Garden, University of Pécs)

3.1.3. Leaf clearing method

For leaf clearing method the dried leaf samples were cut into 1x1 cm pieces (20 pieces/plant). Firstly they were boiled with 15 mL distilled water, 10% ethanol:KOH (15 mL; 7:3) mixture (3-4 min), and 4 mL 5% H_2O_2 (1 min), then they were kept without boiling in 5% H_2O_2 (10 min). After this, samples were washed with distilled water and boiled with 96% ethanol until total decoloration. The samples were again washed with distilled water, and then the colorless pieces were placed onto slides. 10 pieces/species were left as unstained preparations, and 10 ones were stained with 2-3 drops of 0,02% toluidine blue placed with eye-dropper onto the surface of the pieces (5 min). Finally, they were covered with 2-3 drops of Neo-Mount and cover glass. The stained and unstained slides were studied by Nikon Eclipse 80i microscope and SPOT BASIC v4.0 program.

3.1.4. Embedding and preparation of the studied plant parts

The fixed stem, leaf, petiole, immature and mature fruit of each species were dehydrated in acetone series (70% and 90% acetone for 1 hour each, 100% acetone overnight), and in xylol for 2 hours. Samples were embedded in synthetic resin (Technovit 7100) to perform a solid sample-holder block for further section. By a rotary microtome (Anglia Scientific 0325), 10 μm-thick cross sections were prepared from each sample, and longitudinal sections from all fruits. Each sample was placed onto slides and dried at 50°C for 2 hours (exsiccator: Memmert Basic UNB200, 32 L). After this, theye were stained with 0,02% toluidine blue (5 min), stored in distilled water for some seconds, and then they were soaked in 96% ethanol (2x5 min), isopropanol (2 min) and xylol (2x10 min), and finally they were covered with Neo-Mount and cover glass.

Histological features were studied by Nikon Eclipse 80i microscope and SPOT BASIC v4.0, and they were measured by Image Tool 3.00 and Image J programs (20 measurements per plant part of each species). The measured parameters were the following: thickness of cuticle, height of epidermis, palisade and spongy parenchyma cells, and width of vascular bundle in the leaf and the petiole (μm) (PAPP et al. 2013).

3.1.5. Statistical analysis

The micromorphometric data were compared with One-way ANOVA with Tukey's pairwise comparisons. If the normality assumption was violated, we applied Kruskal–Wallis test with Mann–Whitney pairwise comparisons. The normality of data series was checked by using Shapiro-Wilk test. All statistics of micromorphometric data were calculated with Past statistic software, version 2.17b (HAMMER et al. 2001).

3. 2. Results and discussion

3.2.1. Characters of cleared leaves

The venation form was different in the cleared leaf samples of the studied coffees. The leaves of *C. arabica* can be characterized by thick main vascular bundles without small branches among the veins (Fig.6). Those of *C. benghalensis* have short branches (Fig.7), while long branches can be observed anastomised with each other in *C. liberica* (Fig.8). The bean-shaped guard cells of the stomata are surrounded by 3 subsidiary cells in each species (anisocytic stoma, Fig. 6-8).

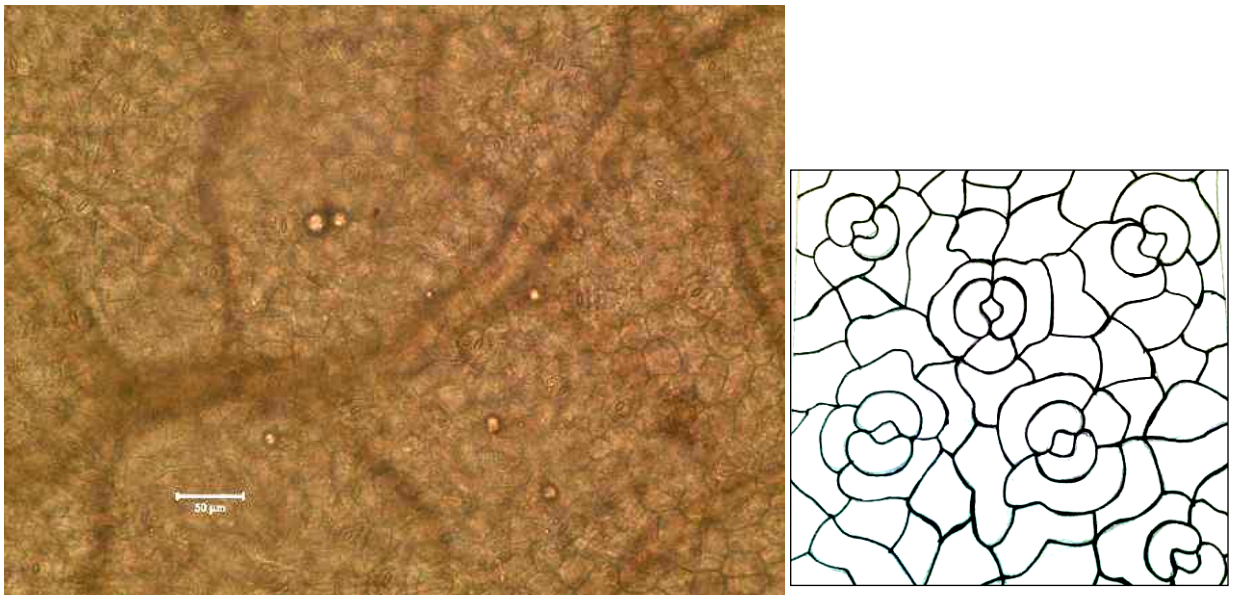

Fig. 6. Cleared leaf and stomata of *C. arabica*

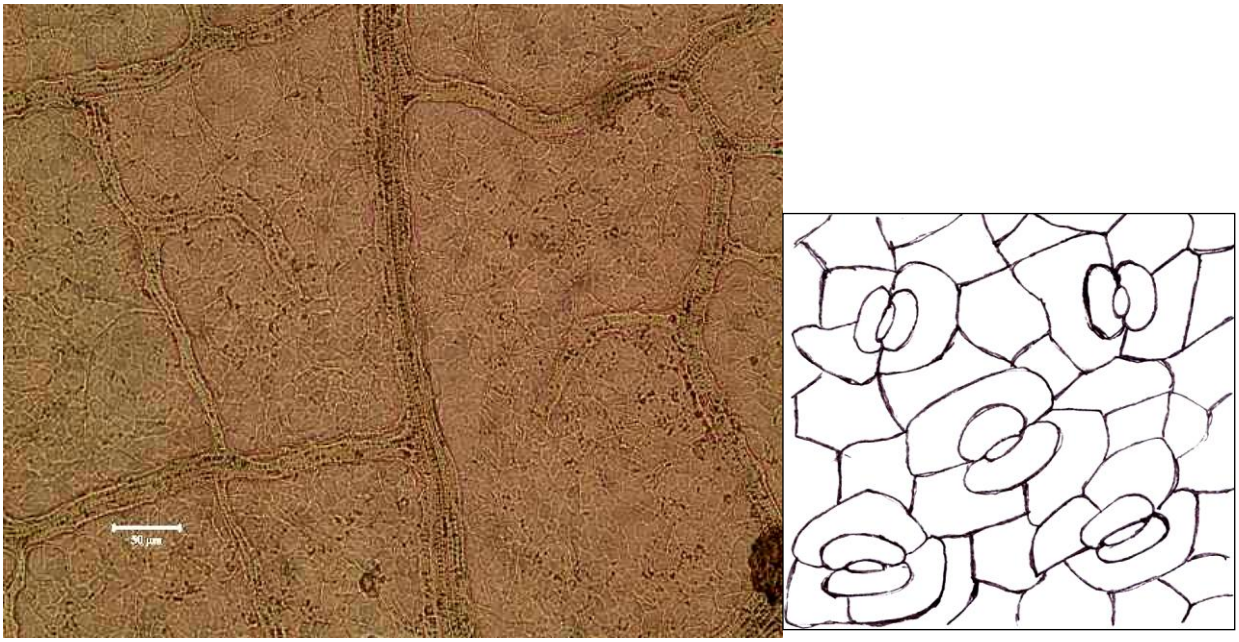

Fig. 7. Cleared leaf and stomata of *C. benghalensis*

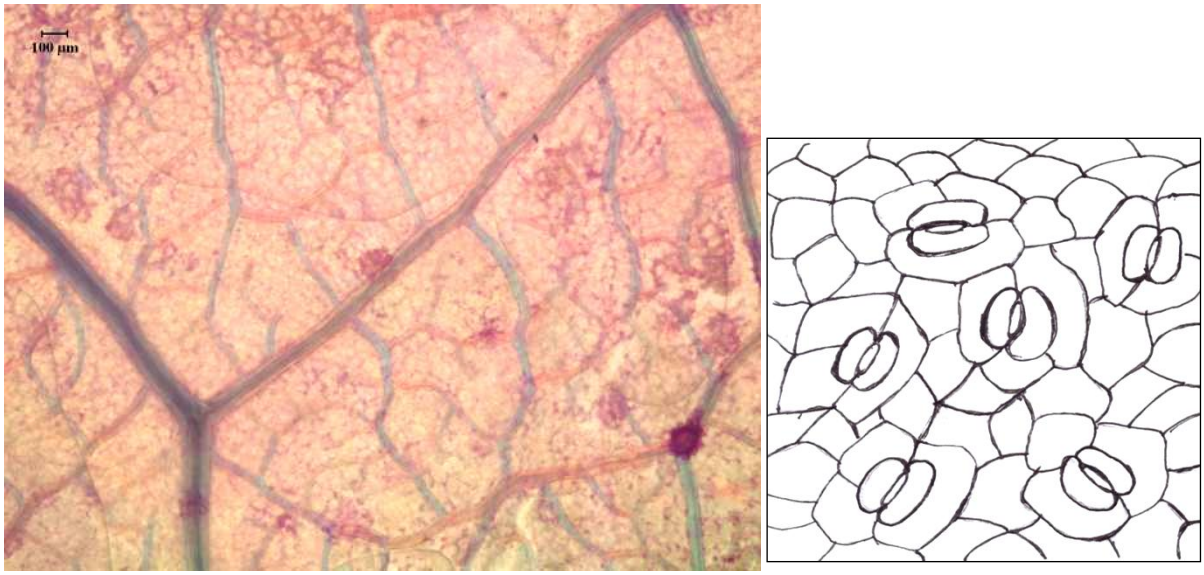

Fig. 8. Cleared leaf and stomata of *C. liberica*

3.2.2. Histological features on preparations

3.2.2.1. Histology of the leaves

The leaf structure of the selected *Coffea* species was similar with some different markers. It is covered by a thin cuticle on the one-cell-layer epidermis of both adaxial and abaxial surfaces in each species (Fig. 9-11) from which the one-cell-layer epidermis of Arabic coffee was reported

earlier (MORAIS et al. 2004). However, the height of both abaxial (26.55 µm) and adaxial (27.18 µm) cells were higher in Arabic coffee, the width of these cells (36.67 and 32.66 µm) was larger in Liberian coffee compared to the other species.

Even though the proportions of epidermal cells of Bengal coffee were not significant, this plant had the thickest cuticle (8.61 and 8.69 µm) among the studied species (PATAY et al. 2016a).

The dorsiventral leaves have heterogenous mesophyll in each plant, which include elongated palisade cells towards the adaxial part and isodiametric spongy parenchyma cells to the abaxial leaf surface (Fig. 9-11). Palisade cells, which contain chloroplasts forming chlorenchyma, extend in a single cell row in Arabic and Liberian similarly to the earlier published features in Arabic coffee (MORAIS et al. 2004), while they can be found in 2 rows in Bengal coffe. The measured parameters of the leaf of Arabic coffee were different compared to an earlier study (FILHO 2006): the height of the adaxial and abaxial cuticle (5.06 and 3.57 µm) and the epidermis cells (26.55 and 27.18 µm) were higher but those of the palisade and spongy parenchyma (30.00 and 31.25 µm) were smaller in our investigation. Among the reasons the different techniques and the various environmental conditions of the samples can be presumably mentioned.

In each species, several intercellular spaces and Ca-oxalate rosette crystals can be found among the spongy cells. However, the height and width of palisade cells (52.77 and 13.88 µm) of Bengal coffee were higher than the other two species, Arabic coffee had the biggest spongy cells (31.25 and 38.20 µm), while Bengal coffee had the widest bundles (267.93 µm) (Fig. 10). Stomata are located in mesomorphic position at the same level with the abaxial epidermis cells in each species. The vascular bundles of each plant consist of phloem and xylem elements composing a collateral closed structure without cambium. At the upper part of the phloem elements of the central veins, sclerenchyma cells surround the bundles in the middle of the leaves in all species (Fig. 9-11 and App/Table 1.)

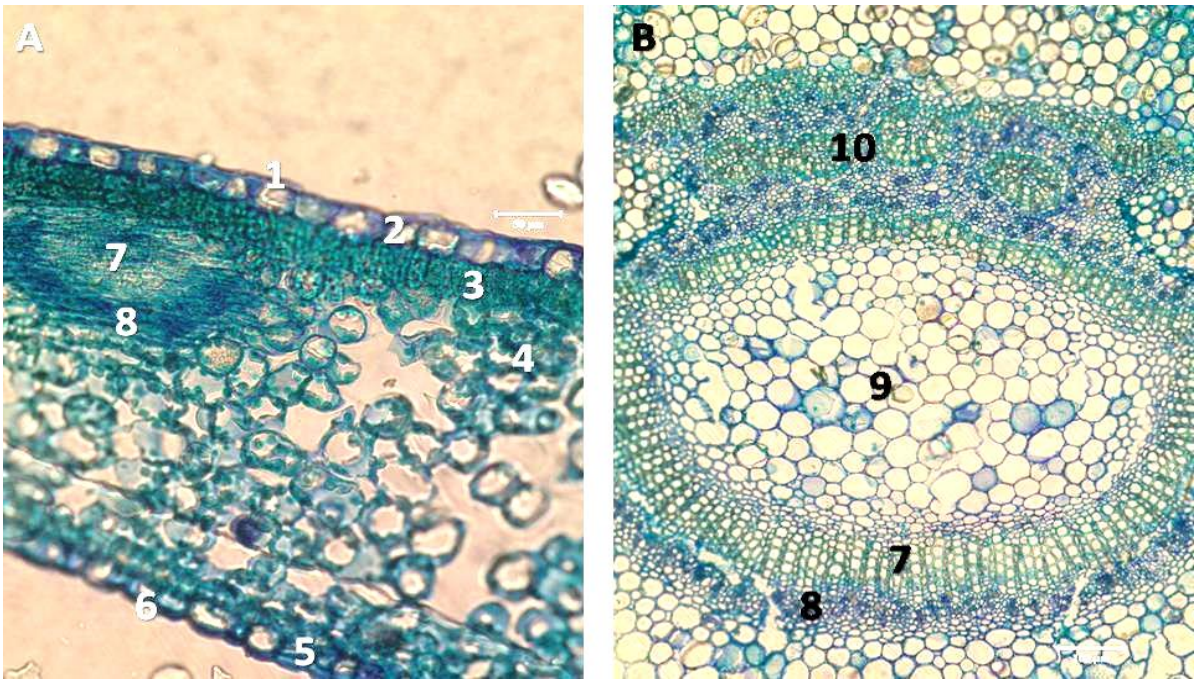

Fig. 9. Histological structure of the leaf blade (A) and the midvein (B) of *C. arabica*

1: adaxial cuticle; 2: adaxial epidermis; 3: palisade parenchyma; 4: spongy parenchyma; 5: abaxial epidermis; 6: abaxial cuticle; 7: xylem; 8 phloem; 9: parenchyma; 10: vascular bundles

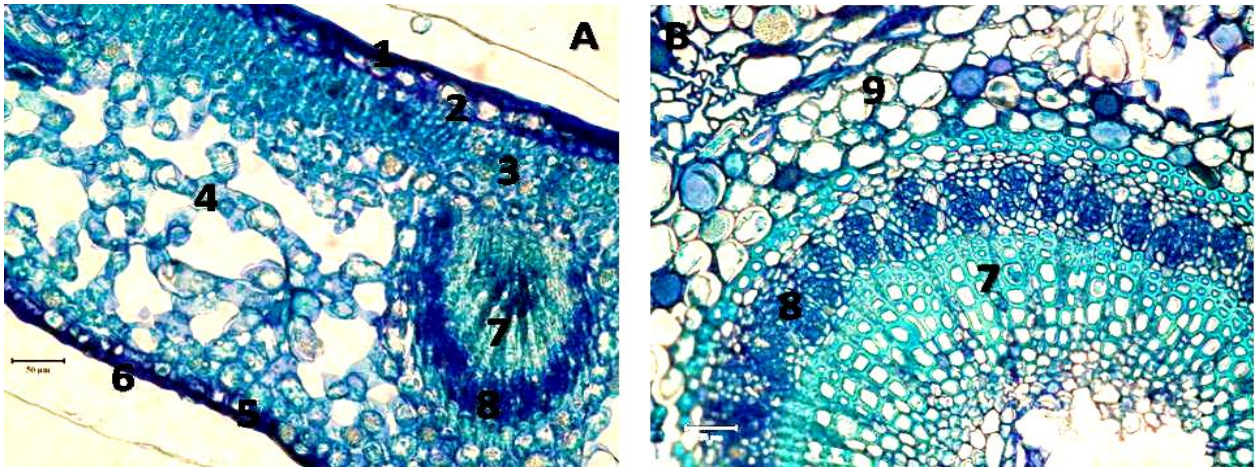

Fig. 10. Histological structure of the leaf blade (A) and midvein (B) of *C. benghalensis*

1: adaxial cuticle; 2: adaxial epidermis; 3: palisade parenchyma; 4: spongy parenchyma; 5: abaxial epidermis; 6: abaxial cuticle; 7: xylem; 8: phloem; 9: parenchyma

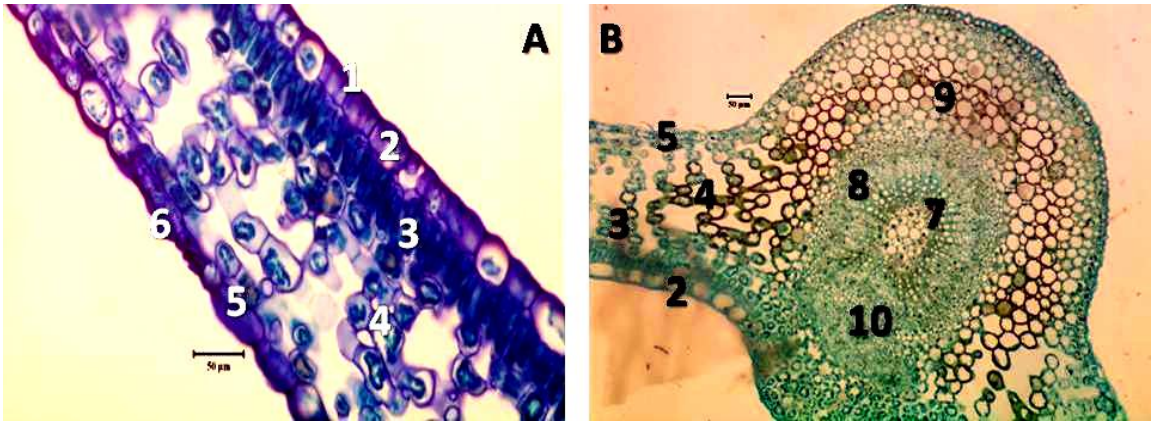

Fig. 11. Histological structure of the leaf blade (A) and midvein (B) of *C. liberica*
1: adaxial cuticle; 2: adaxial epidermis; 3: palisade parenchyma; 4: spongy parenchyma; 5: abaxial epidermis; 6: abaxial cuticle; 7: xylem; 8: phloem; 9: parenchyma; 10: vascular bundles

Statistical analysis

All measured histological parameters of the leaves were statistically analysed (Table 4). Differences were observed in the height of the abaxial epidermis and spongy parenchyma cells of *C. arabica*, while the height of the adaxial epidermis cells was higher than in *C. liberica*. The width of the vascular bundles of Arabic coffe was higher than in *C. benghalensis*. The both hight and width of palisade parenchyma cells and both cuticule thickness were higher in *C. benghalensis* than in the other two species. In addition, the width of abaxial epidermis cells was bigger in *C. liberica* than the other two species.

Table 4. Statistical analysis of the histological values in the leaf of the selected coffees

Measured parameters	***C. arabica* – *C. benghalensis***	***C. arabica* – *C. liberica***	***C. benghalensis* – *C. liberica***
adaxial ep h	↑ *C. arab* ($p<0.001$)	-	↑ *C. lib* ($p<0.001$)
adaxial ep w	-	-	↑ *C. lib* ($p<0.01$)
abaxial ep h	↑ *C. arab* ($p<0.001$)	↑ *C. arab* ($p<0.01$)	-
abaxial ep w	-	↑ *C. lib* ($p<0.001$)	↑ *C. lib* ($p<0.001$)
palisade par h	↑ *C. beng* ($p<0.001$)	-	↑ *C. beng* ($p<0.001$)
palisade par w	↑ *C. beng* ($p<0.01$)	-	-
spongy par h	-	↑ *C. arab* ($p<0.05$)	-
spongy par w	↑ *C. arab* ($p<0.01$)	↑ *C. arab* ($p<0.05$)	-
adaxial cuticle th	↑ *C. beng* ($p<0.001$)	↑ *C. lib* ($p<0.05$)	↑ *C. beng* ($p<0.001$)
abaxial cuticle th	↑ *C. beng* ($p<0.001$)	-	↑ *C. beng* ($p<0.001$)
vascular bundles w	-	↑ *C. arab* ($p<0.01$)	↑ *C. beng* ($p<0.0001$)

Abbreviations: ↑: measured parameter was higher; - : there is no measured difference; h = height; w = width; th = thickness; par = parenchyma; ep = epidermis; arab = *arabica*; beng = *benghalensis*; lib = *liberica*

3.2.2.2. Histology of the petioles

Petioles were similar in their histological features in the studied plants. It is covered by one-cell-layer epidermis in each plant. Collateral closed bundles are surrounded by isodiametric parenchyma and sclerenchymatous cells, which consist of phloem and xylem elements similarly to the vascular bundles of the leaf (Fig. 12-14). The central bundle is circular closing on the adaxial surface of the petiole, which continues in 6 smaller bundles in Arabic, and 4 in Bengal coffee. Central bundle closes with itself in the petiole of Liberian coffee (Fig. 14). Ca-oxalate rosette crystals can be observed among the isodiametric cells in each species. However the measured values of parenchyma cells were similar in Arabic (39.75 and 38.25 µm) and Bengal coffee (32.57 and 38.28 µm), Bengal coffee had the highest epidermis cells (23.17/19.49 and 20.94/18.39 µm) and the widest bundles (287.51 µm) compared with the other species (App/Table 2.)

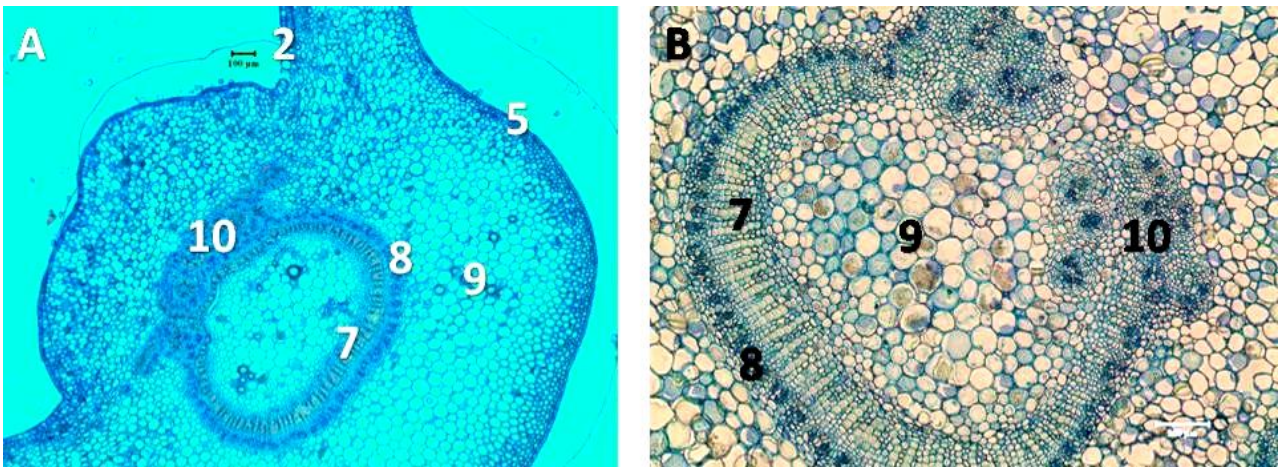

Fig. 12. Histological structure of the petiole (A-B) of *C. arabica*

2: adaxial epidermis; 5: abaxial epidermis; 7: xylem; 8: phloem; 9: parenchyma;

10: vascular bundles

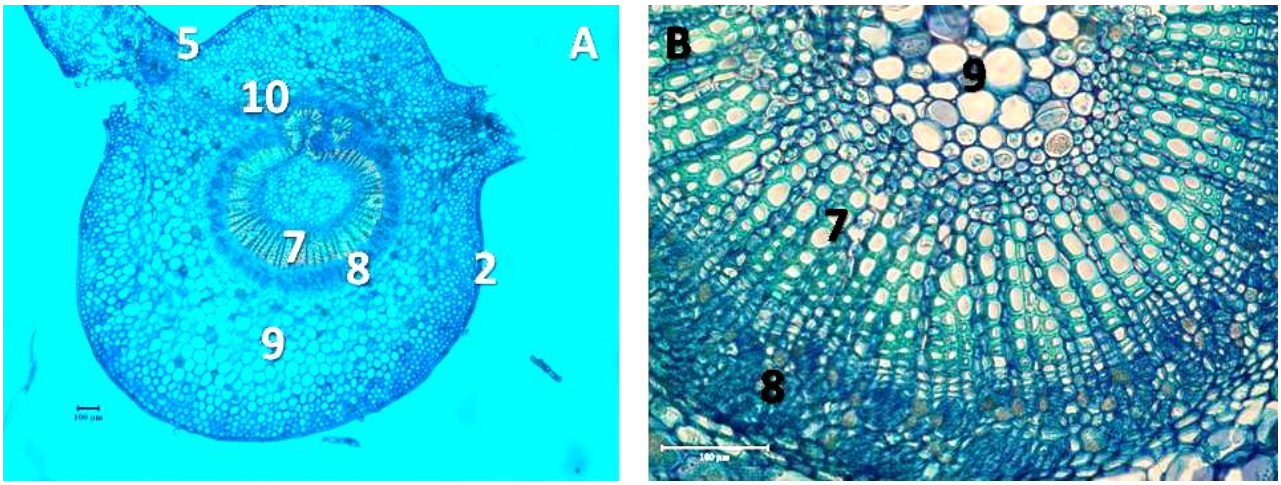

Fig. 13. Histological structure of the petiole (A-B) of *C. benghalensis*

2: adaxial epidermis; 5: abaxial epidermis; 7: xylem; 8: phloem; 9: parenchyma;

10: vascular bundles

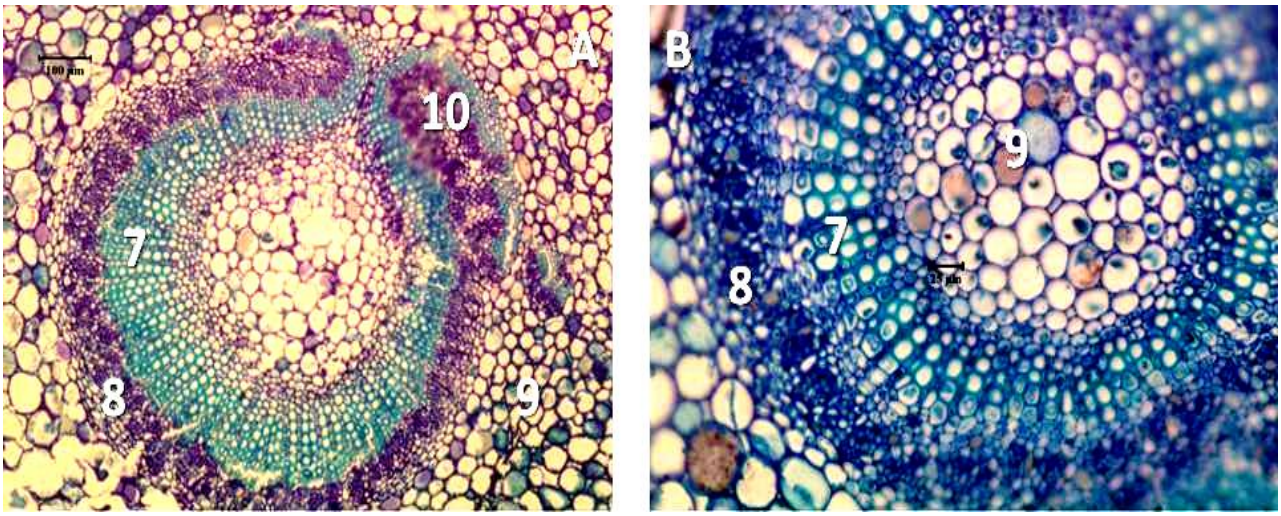

Fig. 14. Histological structure of the petiole (A-B) of *C. liberica*

7: xylem; 8: phloem; 9: parenchyma; 10: vascular bundles

Statistical analysis

All measured histological parameters of the petioles were statistically analysed (Table 5). The petiole of *C. benghalensis* can be described by most statistical differences compared with the other two species. However, the width of adaxial and abaxial epidermis, as well as the adaxial cuticle thickness of *C. benghalensis* was higher than those of *C. arabica* and *C. liberica*, the height of adaxial epidermis, both height and width of parenchyma and the width of vascular bundles of *C. benghalensis* was similar to *C. arabica*. In addition, the width of abaxial epidermis, parenchyma and cuticle thickness measured in the previous species were similar to those of *C. liberica*.

Table 5. Statistical analysis of the histological values in the petiole of the selected coffees

Measured parameters	***C. arabica* – *C. benghalensis***	***C. arabica* – *C. liberica***	***C. benghalensis* – *C. liberica***
adaxial ep h	-	↑ *C. arab* ($p<0.01$)	↑ *C. beng* ($p<0.001$)
adaxial ep w	↑ *C. beng* ($p<0.001$)	-	↑ *C. beng* ($p<0.001$)
abaxial ep h	↑ *C. beng* ($p<0.05$)	↑ *C. arab* ($p<0.001$)	↑ *C. beng* ($p<0.001$)
abaxial ep w	↑ *C. beng* ($p<0.05$)	↑ *C. lib* ($p<0.01$)	-
par h	-	↑ *C. arab* ($p<0.001$)	↑ *C. beng* ($p<0.001$)
par w	-	↑ *C. arab* ($p<0.01$)	-
adaxial cuticle th	↑ *C. beng* ($p<0.001$)	↑ *C. lib* ($p<0.01$)	↑ *C. beng* ($p<0.001$)
abaxial cuticle th	↑ *C. beng* ($p<0.001$)	↑ *C. lib* ($p<0.001$)	-
vascular bundles w	-	↑ *C. arab* ($p<0.01$)	↑ *C. beng*($p<0.001$)

Abbreviations: ↑: measured parameter was higher; - : there is no measured difference; h = height; w = width; th = thickness; par = parenchyma; ep = epidermis; arab = *arabica*; beng = *benghalensis*; lib = *liberica*

3.2.2.3. Histology of the stems

Similar to the leaf and the petiole, the stem is covered by one-cell-layer epidermis in each plant (Fig. 15-17). The cortex consisting of parenchyma cells is thicker in Arabic coffee (10-12 cell rows), than in Bengal (7-8 rows) and Liberian coffee (6-8 rows). These cells are isodiametric in Arabic and Liberian, also isodiametric in the outer 5-6 rows, but flattened in the internal rows in Bengal coffee. The vascular elements form a concentric ringle in the middle of the stem which is surrounded by one-cell-layer sclerenchyma in each plant. Among the phloem and xylem elements a thin cambium layer extend. The medullary part in the middle of the stems is filled with isodiametric parenchyma cells in all coffees. Even though Bengal coffee presented the highest epidermis cells (16.41 and 17.39 µm), Liberian coffee can be described with the biggest parenchyma cells (43.64 and 46.21 µm) in the stem (App/Table 3.).

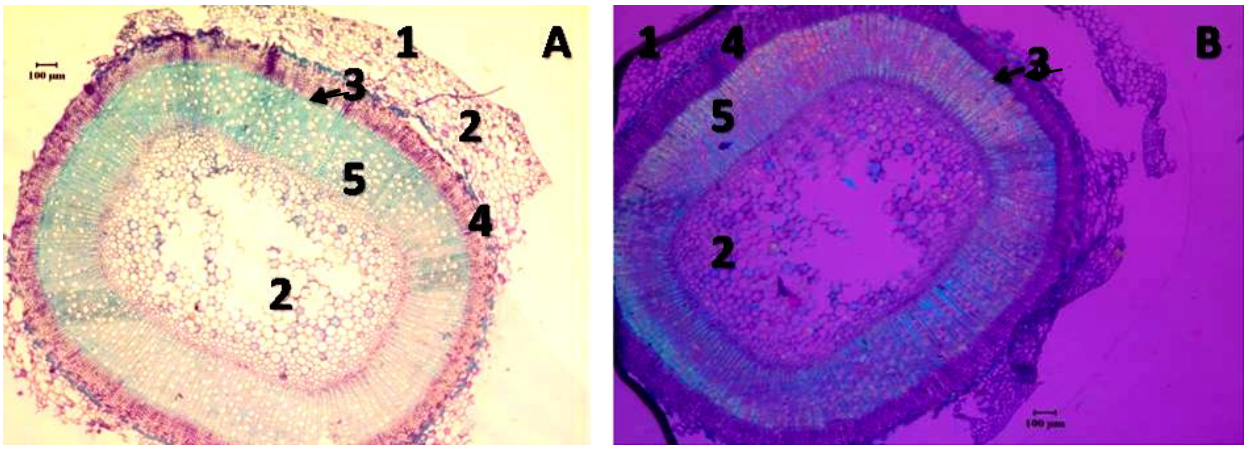

Fig. 15. Histological structure of the stem (A-B) of *C. arabica*

1: epidermis; 2: parenchyma; 3: cambium; 4: phloem; 5: xylem

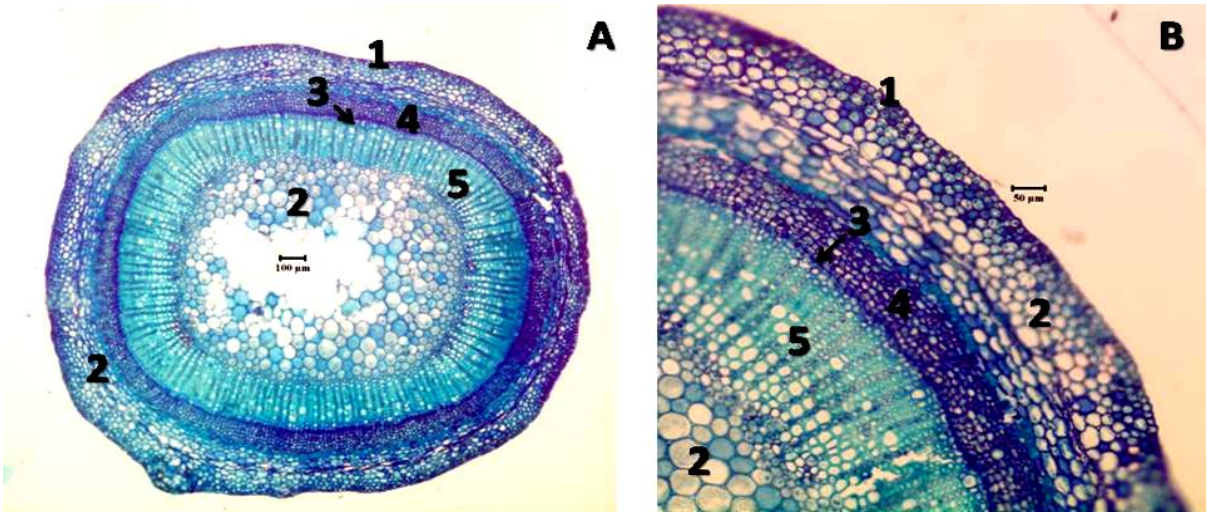

Fig. 16. Histological structure of the stem (A-B) of *C. benghalensis*

1: epidermis; 2: parenchyma; 3: cambium; 4: phloem; 5: xylem

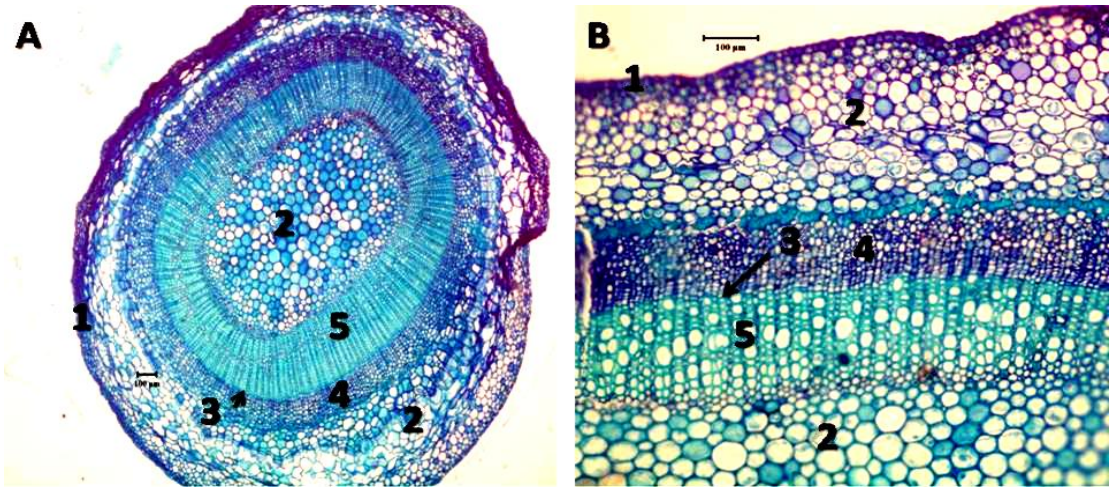

Fig. 17. Histological structure of the stem (A-B) of *C. liberica*

1: epidermis; 2: parenchyma; 3: cambium; 4: phloem; 5: xylem

Statistical analysis

All measured histological parameters of the stems were statistically analysed (Table 6). However, both the height and width of the parenchyma cells were the highest in *C. liberica*, the epidermis cells were highest in Bengal coffee. Statistical differences were not observed in the width of epidermis cells of the studied species.

Table 6. Statistical analysis of the histological values in the stem of the selected coffees

Measured parameters	***C. arabica* – *C. benghalensis***	***C. arabica* – *C. liberica***	***C. benghalensis* – *C. liberica***
epidermis h	↑ *C. beng* ($p<0.001$)	↑ *C. arab* ($p<0.01$)	↑ *C. beng* ($p<0.001$)
epidermis w	-	-	-
par h	↑ *C. arab* ($p<0.05$)	↑ *C. lib* ($p<0.001$)	↑ *C. lib* ($p<0.001$)
par w	-	-	↑ *C. lib* ($p<0.01$)

Abbreviations: ↑ : measured parameter was higher; - : there is no measured difference; h = height; w = width; th = thickness; par = parenchyma; ep = epidermis; arab = *arabica*; beng = *benghalensis*; lib = *liberica*

3.2.2.4. Histology of the fruits

The fruit includes 2 bean-shaped seeds in each species which turn to each other with their flattened side. The pericarps are covered by one-cell-layer epidermis. The majority of the parenchyma cells are isodiametric, the collateral closed bundles can be found around the seeds in each plant (Fig.

19-23). The seeds of Bengal coffee are surrounded by elongated sclereids in 2-3 rows which can be observed better in fluorescent light (Fig. 21). However, the size of epidermal cells were similar in both Arabic (11.22 and 15.07 µm) and Bengal coffee (17.22 and 13.35 µm), parenchyma cells were bigger in Bengal coffee (28.03 and 51.67 µm) than the other plants (App/Table 4.).

Due to the fruits had large size and dimensions, the structure of the whole cross sections could not be completely observed under microscope, therefore the preparations were documented by macroscopy (Fig. 18,20,22).

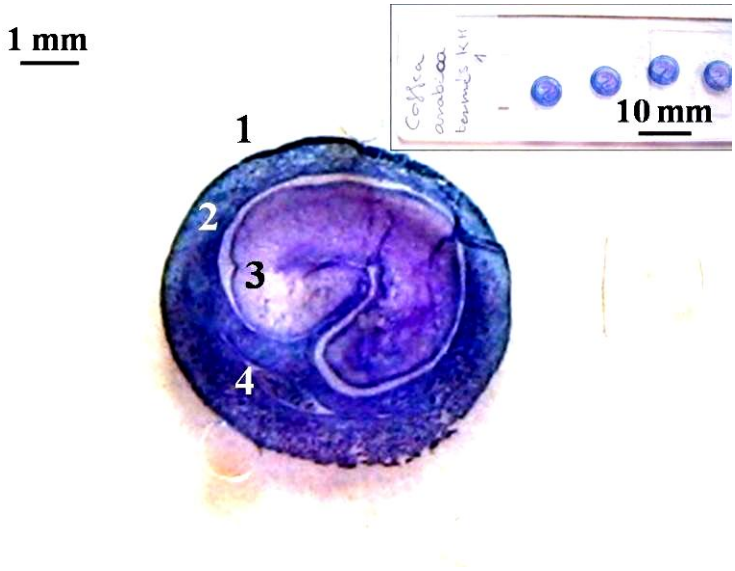

Fig. 18. Preparation detail of the fruit cross section of *C. arabica*

1: epicarp of pericarp (epidermis); 2: parenchyma cells; 3: developed seed; 4: chalaza

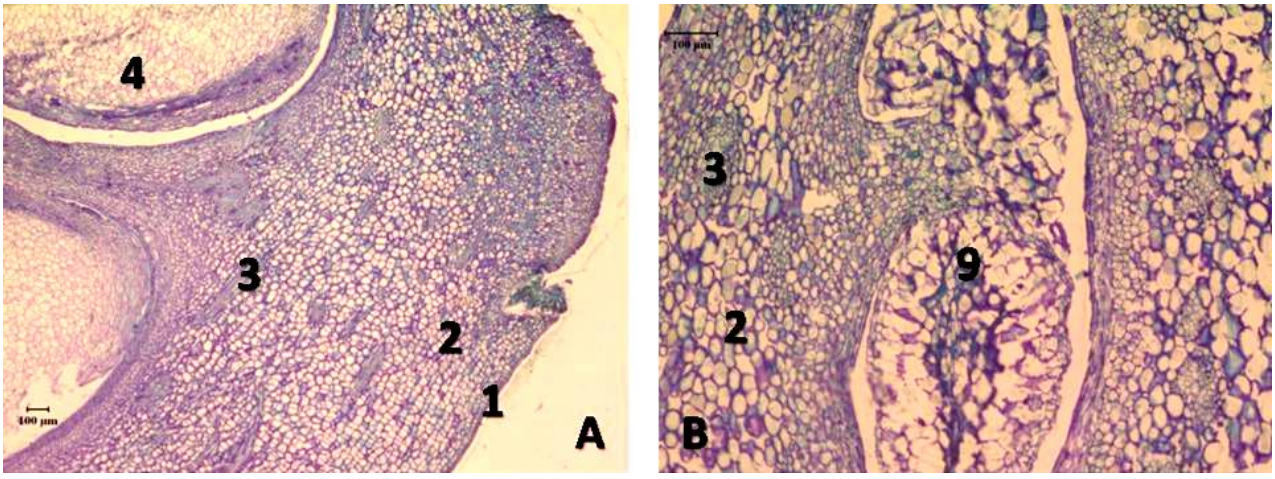

Fig. 19. Histological structure of the fruit (A-B) of *C. arabica*

1: epidermis; 2: parenchyma; 3: vascular bundles; 4,9: developed seed

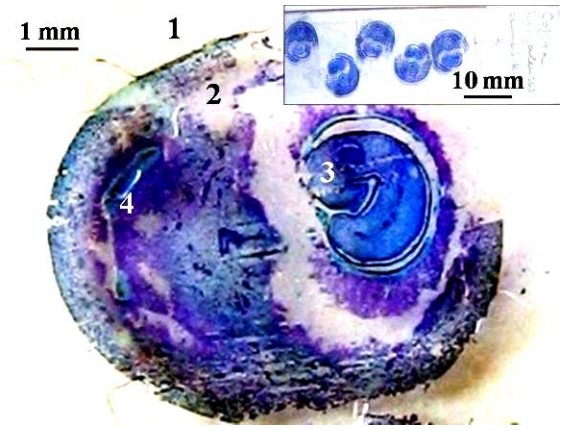

Fig. 20. Preparation detail of the fruit cross section of *C. benghalensis*

1: epicarp of pericarp (epidermis); 2: parenchyma cells; 3,4: developed seed

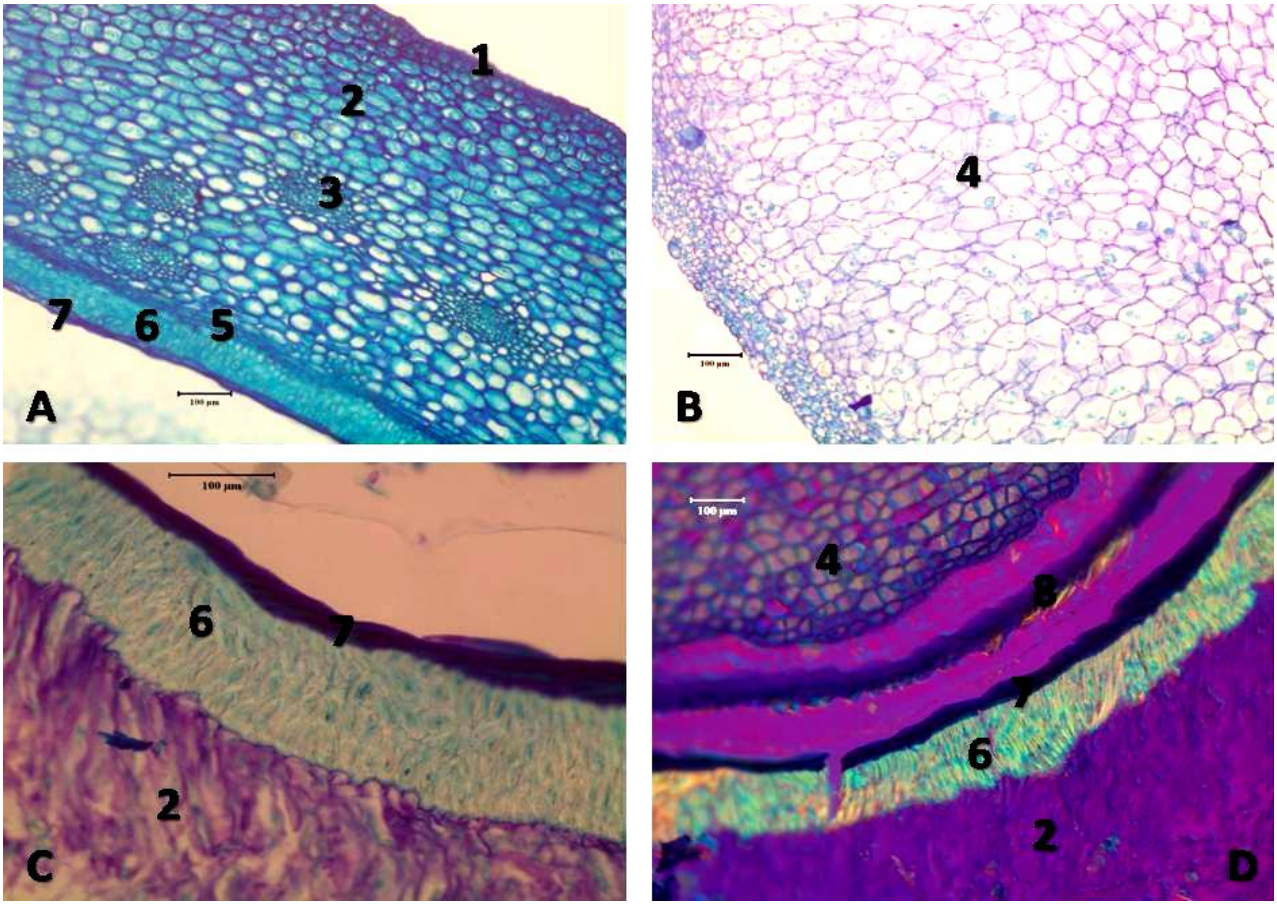

Fig. 21. Histological structure of the fruit (A-D) of *C. benghalensis*

1: epidermis; 2: parenchyma; 3: vascular bundles; 4: developed seed; 5: sclerencyma cells; 6: sclereids; 7-8: parenchyma cells

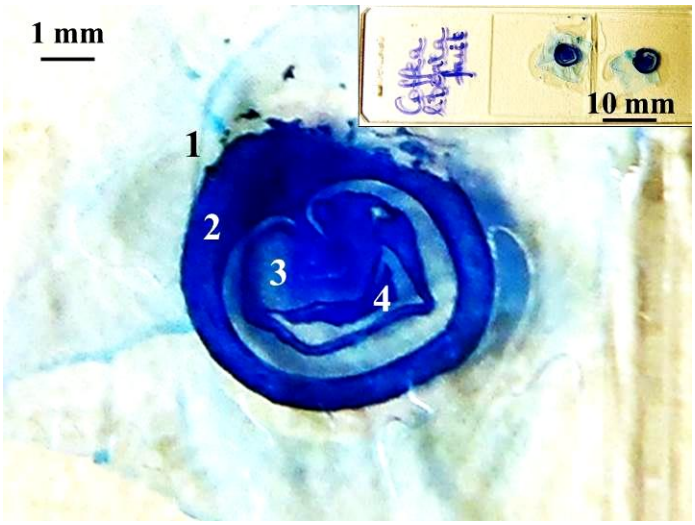

Fig. 22. Preparation detail of the fruit cross section of *C. liberica*

1: epicarp of pericarp (epidermis); 2: parenchyma cells; 3: developed seed; 4: chalaza

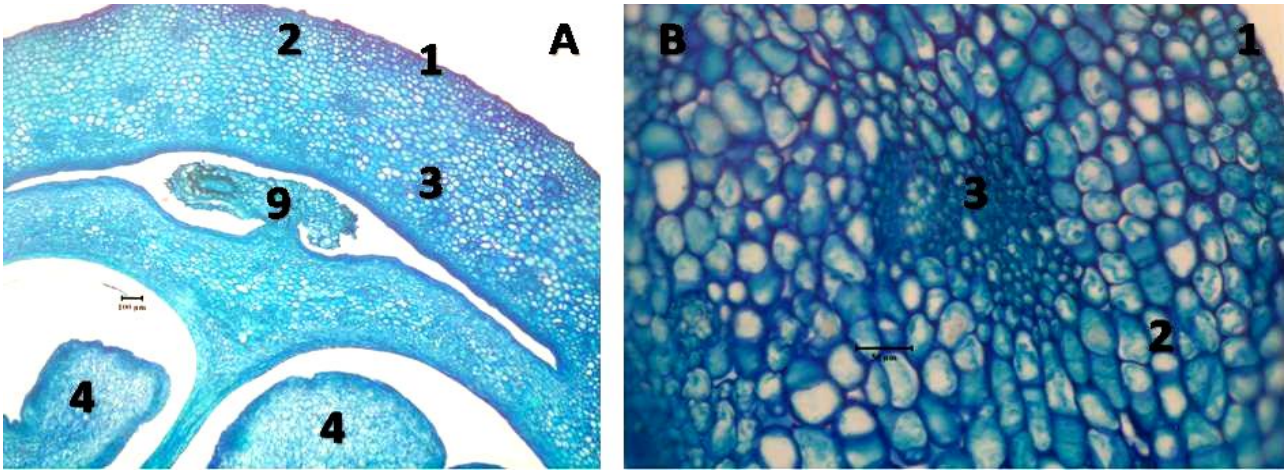

Fig. 23. Histological structure of the fruit (A-D) of *C. liberic*

1: epidermis; 2: parenchyma; 3: vascular bundles; 4: developed seed; 9: chalaza

Statistical analysis

All measured histological parameters of the fruits were statistically analysed (Table 7). However, the epidermis cells were more higher in *C. benghalensis* and *C. liberica* than in *C. arabica*, the width of parenchyma cells was the highest in *C. benghalensis.* Statistical differences were not observed in the width and height of epidermis cells in the studied species.

Table 7. Statistical analysis of the histological values in the fruit of the selected coffees

Measured parameters	***C. arabica – C. benghalensis***	***C. arabica – C. liberica***	***C. benghalensis – C. liberica***
epidermis h	↑ *C. beng* ($p<0.001$)	↑ *C. lib* ($p<0.001$)	-
epidermis w	-	-	-
par h	-	-	-
par w	↑ *C. beng* ($p<0.001$)	-	↑ *C. beng* ($p<0.001$)

Abbreviations: ↑: measured parameter was higher; - : there is no measured difference; h = height; w = width; th = thickness; par = parenchyma; ep = epidermis; arab = *arabica*; beng = *benghalensis*; lib = *liberica*

3.2.3. Conclusions

In the leaf of Bengal coffee, higher palisade cells extended in 2 cell rows and a thicker cuticle, the highest epidermis cells of the petiole and the stem, and the widest and highest parenchyma cells of the fruit were observed compared to *C. arabica* and *C. liberica.* The detected histological features of the studied *Coffea* species, especially those of *C. benghalensis* add new scientific data to the available records of the plant. Compared with the commonly known data of *C. arabica* and *C. liberica,* our results confirm the significant role of taxonomical description mostly for the identification of less studied Bengal coffee.

4. Phytochemical study of the selected *Coffea* species

Coffees are rich in polyphenols which are a deeply studied group of plant compounds. They present a significant importance for science and the human body. The group of polyphenols includes many subclasses which are illustrated in App/Fig.1.

Nowadays, the most important and known phenolic acids of coffees are chlorogenic acids which are esters formed by quinic acid esterification with trans-cinnamic acids (e.g. caffeic, *p*-coumaric, and ferulic acids). The major kinds of chlorogenic acids are caffeoylquinic, dicaffeoylquinic, feruloylquinic, *p*-coumaroylquinic and caffeoylferuloylquinic acids, in addition, diferuloylquinic, di-*p*-coumaroylquinic and dimethoxycinnamoylquinic acids were also identifed.

Phenolic compounds can be determined by simple but nonspecific spectrophotometric assays like Folin-Denis or Folin-Ciocalteu methods, and by different selective analytical techniques as high-performance liquid chromatography (HPLC). For determinations of structure of separated compounds, mass spectrometry (MS) or tandem mass spectrometry (MS–MS) can be applied. In this way, HPLC method combined with MS one can be used to identify and quantify phenolic compounds of various origin (SAMANIDOU 2015).

As the official used drug is *Coffeae semen*, the most analytical studies are focused on chemical composition of mature and immature fruit of coffees. The aim of our work was to investigate the polyphenol content of the leaf, immature and mature fruit (pericarp and seed) of *C. arabica, C. benghalensis* and *C. liberica*. We were courious if the other plant parts can also been used by the science and the industry besides the widely applied seeds. In addition, we aimed to emphasize that wild coffee species could be as an interesting taxon for science as the well known and used species. The phytochemical features of the selected species were studied by HPLC/ESI/MS, HPLC/ESI/MS/MS, total polyphenol, tannin and Folin–Ciocalteu methods. Firstly only the leaves and immature fuits of *C. arabica* and *C. benghalensis* were studied by HPLC-ESI/MS. After that owing to the promising results we decided to expand the analysis with new methods and plant samples including *C. liberica*. Finally we realised a comprehensive phytochemical study of leaves, immature/mature pericarp and immature/mature seeds of the selected three *Coffea* species using 4 analitical methods.

4.1. Materials and methods

The investigations were performed at the Department of Pharmaceutical Technology and Biopharmaceutics, Faculty of Pharmacy, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca (HPLC-ESI/MS), at the Department of Pharmacognosy, Faculty of Pharmacy, Semmelweis University, Budapest (HPLC-ESI-MS/MS), and at the Department of Pharmacognosy, Faculty of Pharmacy, University of Pécs (total polyphenol, tannin and Folin–Ciocalteu method).

4.1.1. Chemicals

The following chemicals were used for the analyses: ethanol (Merck), petroleum ether (Molar Chemicals Ltd., Budapest, Hungary), methanol of analytical grade (Reanal, Budapest, Hungary), acetic acid and methanol of HPLC supergradient grade (Sigma-Aldrich, Steinheim, Germany). All aqueous eluents for LC-MS were filtered through MF-Millipore membrane filters (0.45 μm, mixed cellulose esters; Billerica, MA, USA), as well as $AlCl_3$ (Alfa Aesar), acetone, 25% HCl, ethyl acetate, 5% methanol-acetic acid (methanol and acetic acid separately from Molar Chemicals Ltd.), distilled water, solution of sodium carbonate (Lach-Ner), phosphor-molybdo-tungstic reagent (Sigma-Aldrich), holystone (Reanal), hide powder, hexamethylene tetramine, and pyrogallol (VWR) were applied.

4.1.2. Sample preparation

The plant samples of *C. arabica, C. benghalensis* and *C. liberica* were collected in the Botanical Garden, University of Pécs in the spring of 2014 and 2015. Voucher specimens of each species were deposited and labelled with unique codes at the Department of Pharmacognosy, University of Pécs. Samples were collected from 2 plants per each species.

For HPLC-ESI/MS studies, 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 in the case of *C. arabica* and *C. benghalensis*. For the hydrolysis the extracts were diluted with HCL 2N 1:1 and they were kept at 80 ºC in water bath for 60 min. The hydrolised extract was supplemented with distilled water for further study.

For HPLC-ESI-MS/MS analyses, all samples were powdered and extracted using Soxhlet-extraction method (70:30 v/v% methanol:distilled water). After evaporation of the solvent the

residues were redissolved in 5 mL 70:30 v/v% methanol-water mixture. Apolar compounds were removed by liquid-liquid extraction with petroleum ether if needed. The seed and pericarp extracts were used until the appearance of the opalescence. Prior to evaluation, all samples were submitted to SPE purification (500 mg/3mL Supelco Supelclean LC-18 SPE cartridges, Sigma-Aldrich, Steinheim, Germany) and were filtered through Sartorius (Goettingen, Germany) Minisart RC15 syringe filters (0.2 μm). The extractions were carried out with 150 mL solvent lasted 8 h. Chlorogenic acid was used as standard compound and the other compounds were identified by data of scientific literature. The used samples, extracts and injected extract quantities are illustrated in the App/Table 6.

For the study of total polyphenol and tannin content, 0.5 g powdered immature and mature pericarp and seed of each species were mixed with 150 mL distilled water, and then they were heated on water-bath for 30 min at 70°C. The cooled extracts were transferred quantitatively to a 250 mL volumetric flask, then they were filtrated and used for the reactions.

For the study of total flavonoid content, 0.5 g powdered samples were mixed with 1 mL of 0.5% hexamethylene tetramine, 20 mL acetone, 2 mL of 25% HCl, and holystone. The mixtures were stored on reflux condenser for 30 min and shaken with distilled water and ethyl acetate in separation funnel.

4.1.3. HPLC-ESI/MS

Polyphenolic compounds were determined by LC/MS which is based on earlier analyses (SUCIU et al. 2004a, VLASE et al. 2005, CRIȘAN et al. 2010, FODOREA et al. 2005a, FODOREA et al. 2003, SUCIU et al. 2004b, FODOREA et al. 2005b) however, some modifications were added to them in our work. The major one is the changing of the components of mobile phase (acetic acid instead of potassium phosphate). The reason of this is to obtain supplementary information about polyphenolic compounds. Due to overlapping of caffeic acid with chlorogenic acid and caftaric acid with gentisic acid, they were determined qualitatively by LC/MS/MS method as the UV identification was not possible. The calibration curves of caffeic and chlorogenic acid are illustrated in the Appendix (App/Fig. 15,16).

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, 100x3.0 mm i.d., 3.5 µm 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 by an electrospray ion source in negative mode. The chromatographic data were processed using Chem Station and Data Analysis software (Agilent, USA). The mobile phase was a binary gradient prepared from methanol and solution of acetic acid 0.1% (v/v). The gradient method was: 0 min 5 v/v% methanol, 35 min 42 v/v% methanol, 38 min 42 v/v% methanol, 45 min 5 v/v% methanol – rebalancing.

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 (App/Fig.2). These were the followings: caftaric, gentistic, caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic acids, as well as hyperoside, rutoside, myricetin, fisetin, quercitrin, quercetin, patuletine, hyperoside, isoquercitrin, kaempherol, apigenin, and luteolin (App/Table 5). A signal was also observed at t_R =12.5 which was presumably *cis*-ferulic acid 6' noted by 6' in the App/Table 1. This compound forms through isomerisation from ferulic acid.

4.1.4. HPLC-ESI-MS/MS

Chromatographic analyses were performed on an Agilent 1100 HPLC Series system coupled with an Agilent 6410 Triple Quadrupole mass spectrometer using an electrospray ionsource in negative ionization mode. For separation, a ZORBAX SB-C18 3.0x150 mm, 3.5 µm column was used. As mobile phase A and B, 0.3 v/v% acetic acid in water and methanol were used respectively with a gradient method as follows: 0 min 10 v/v% B, 30 min 100 v/v% B, 35 min 100 v/v% B. The temperature of the column was kept at 25°C. The flow rate of the mobile phase was 0.3 ml/min and the injection volume was 5 µL. ESI conditions were as follows: temperature: 350°C, nebulizer pressure: 40 psi (N_2), drying gas flow rate: 9 l/min (N_2), capillary voltage: 3500 V, fragmentor voltage: 100 V; according to structural differences, collision energy was changed between 10-50 eV. High purity nitrogen was used as collision gas. Full mass scan spectra were recorded over the range *m/z*70-1000 (1 scan/sec). Masshunter B.01.03 software was used for data acquisition and qualitative analysis. For unambiguous identification, retention times, UV and mass spectra were

compared with literature data and with those of authentic standards (DONGMEI et al. 2008, SUN et al. 2009, TOTH et al. 2015, RIETHMÜLLER et al. 2013, PERLATTI et al. 2014, MOEENFARD et al. 2014).

4.1.5. Total polyphenol and tannin content

The powdered samples were heated with 15.0 mL distilled water for 30 min. The cooled extracts were diluted to 25.0 mL with distilled water, then they were filtrated and the first 50 mL was discarded from each extract.

a. Total polyphenols

This method is based on EUROPEAN PHARMACOPOEIA 7th ed (2010). 5.0 mL of the filtrate was diluted to 25.0 mL with distilled water. 2.0 mL of the solution was mixed with 1.0 mL of phosphor-molybdo-tungstic reagent and 10.0 mL of distilled water, then it was diluted to 25.0 mL with a 290 g/L solution of sodium carbonate. After 30 min, the absorbance was measured at 760 nm (*A1*) against distilled water by Metertech UV/VIS SP8001 Spectrophotometer.

b. Polyphenols not adsorbed by hide powder

10 mL of the filtrate was mixed with 0.10 g of hide powder and shaken for 60 min. 5.0 mL of the filtrate was diluted to 25.0 mL with distilled water, then 2.0 mL of this solution was mixed with 1.0 mL of phosphor-molybdo-tungstic reagent and 10.0 mL of distilled water. Then the mixture was diluted to 25.0 ml with a 290 g/L solution of sodium carbonate. After 30 min, the absorbance was measured at 760 nm (*A2*) against distilled water.

c. Pyrogallol standard solution for polyphenol content

50.0 mg of pyrogallol was dissolved in distilled water and diluted to 100.0 mL with the same solvent. 5.0 mL of the solution was diluted to 100.0 mL with distilled water, then 2.0 mL of this solution was mixed with 1.0 mL of phosphor-molybdo-tungstic reagent and 10.0 mL water. This mixture was diluted to 25.0 ml with a 290 g/L solution of sodium carbonate. After 30 min, the absorbance was measured at 760 nm (*A*3) against distilled water.

Each analysis was performed in duplicate. Polyphenol contents were calculated with the following formulas (EUROPEAN PHARMACOPOEIA 7th ed 2010):

Polyphenols not adsorbed by hide powder: $[62.5 \cdot (A_1 - A_2) \cdot m_2] / A_3 \cdot m_1$

$m1$ = mass of the sample to be examined in grams

$m2$ = mass of pyrogallol in grams

Total polyphenols: $[62.5 \cdot A_1 \cdot m_2]/A_3 \cdot m_1$

$m1$ = mass of the sample to be examined in grams

$m2$ = mass of pyrogallol in grams

4. 2. Results and discussion

4.2.1. Compounds detected by HPLC-ESI/MS

Detection and evaluation of polyphenols

These analyses were carried out on the leaf, immature pericarp and seed samples of *C. benghalensis* and *C. arabica*.

Nine polyphenolic compounds involving 5 phenolic acids (caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, sinapic acid), and 4 flavonols (quercetin, isoquercetin, kaempherol, rutin) were identified and quantified in Bengal coffee (Table 8-10, App/Fig. 3-14, App/Table 12). Phenolic acids were identified also in the leaf, immature pericarp and seed, but flavonols appeared only in the leaf and the immature pericarp of the species. Some differences were found in the non-hydrolysed and hydrolised extracts of the leaf 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 hydrolised extracts, caffeic acid was detected in a large amount in the leaf, but kaempherol was observed only at a low level in the immature pericarp. Phenolic acids (caffeic and ferulic acid) were found in largest amount in the hydrolised leaf extract, chlorogenic acid in the non-hydrolysed and *p*-coumaric acid in the hydrolised extracts of the immature seed (Table 10).

Among the identified flavonols of the leaf in both species, isoquercitrin and rutin were detected as dominant compounds in the non-hydrolysed extract, while quercetin and kaempherol in hydrolised ones (PATAY et al. 2014b). In earlier reports, according to the protective role of polyphenol, the amounts of total phenols in developing coffee species were the same in the leaves of fruit producing and non-producing plants (SALGADO et al. 2008).

In the immature pericarp, each selected compound was represented at a low level (Table 8). The detected lower level of polyphenols of the immature pericarp is due presumably to various environmental factors which were not studied in this work.

Compared the identified and quantified polyphenols of *C. benghalensis* and *C. arabica* (Table 8-10), some differences were found in the values of chlorogenic, ferulic, *p*-coumaric and sinapic acid

which occur in larger amount in the leaf and the seed of Arabic than in Bengal coffee, however, sinapic acid was observed only in the leaf of Arabic coffee. In contrast, flavonols occur at a higher level in the leaf of *C. benghalensis* than of *C. arabica*.

Table 8. Polyphenolic compounds of the leaf of *C. benghalensis* and *C. arabica*

Proposed compound	**UV identi-fication**	***C. benghalensis***		***C. arabica***	
		Non-hydrolysed extract (µg/mL)	**Hydrolysed extract (µg/mL)**	**Non-hydrolysed extract (µg/mL)**	**Hydrolysed extract (µg/mL)**
Caffeic acid	–	2.26	125.55	1.82	86.52
Chlorogenic acid	–	428.73	-	605.65	-
p-Coumaric acid	+	-	0.80	0.86	1.10
Isoquercitrin	+	7.439	-	6.36	-
Rutin	+	12.88	-	1.59	-
Ferulic acid	+	-	8.49	-	103.89
Sinapic acid	+	-	7.34	-	8.20
Quercetin	+	-	42.73	-	11.95
Kaempferol	+	-	5.72	-	1.54

Abbreviations: compounds were determined (+) or not (-) with UV identification

Table 9. Polyphenolic compounds of the immature pericarp of *C. benghalensis* and *C. arabica*

Proposed compound	**UV identi-fication**	***C. benghalensis***		***C. arabica***	
		Non-hydrolysed extract (µg/mL)	**Hydrolysed extract (µg/mL)**	**Non-hydrolysed extract (µg/mL)**	**Hydrolysed extract (µg/mL)**
Caffeic acid	-	1.89	49.11	1.46	41.22
Chlorogenic acid	-	211.68	-	297.82	-
p-Coumaric acid	-	-	0.26	-	0.56
Isoquercitrin	+	6.82	-	7.28	-
Rutin	+	0.41	-	1.15	-
Ferulic acid	-	-	1.61	-	3.23
Sinapic acid	+	-	0.49	-	-
Quercetin	+	-	1.55	-	1.55
Kaempferol	+	-	0.22	-	0.25

Abbreviations: compounds were determined (+) or not (-) with UV identification.

Table 10. Polyphenolic compounds of the immature seed of *C. benghalensis* and *C. arabica*

Proposed compound	UV identi-fication	*C. benghalensis*		*C. arabica*	
		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-Coumaric acid	+	-	1.28	-	2.30
Ferulic acid	+	-	8.49	-	11.17
Sinapic acid	+	-	0.66	-	-

Abbreviations: compounds were determined (+) or not (-) with UV identification

4.2.2. Compounds detected by HPLC-ESI-MS/MS

These analyses were performed in all samples of each selected coffee species.

Altogether 25 phenolic components were identified in the extracts of the studied parts of the *Coffea* species (Table 11), among which 22 compounds were found in Bengal coffee. Among them, 16 phenolic acid derivatives (e.g. caffeoylquinic acids), 2 flavan-3-ols, 2 procyanidin dimers and 2 procyanidin trimers, a xanthonoid, and 2 aliphatic tricarboxylic acids were qualitatively characterized by comparison of their LC-ESI-MS/MS data with the literature and mass spectral data of reference compounds (App/Table 7-11).

Phenolic compositions of the studied coffee species were similar with minor differences. Chlorogenic acids were observed as main components in each extract. 4-caffeoylquinic acid (4-CQA) and 5-caffeoylquinic acid (5-CQA) were detected in each sample, except the latter was missing in *C. arabica* leaf extract. Dicaffeoylquinic acids (diCQAs) were characteristic to most extracts, 4,5-diCQA (24) was present in all samples, except Bengal coffee immature pericarp, while 3,4-diCQA (22) was detected in each sample excluding the mature pericarp of Liberian coffee and the immature pericarp of Bengal coffee (CLIFFORD et al. 2003, JAISWAL et al. 2014). In addition, isocitric acid was described in all samples (BYLUND et al. 2007). The most complex composition including 17 compounds was detected in the immature pericarp of Arabic coffee, followed by the extract of the mature pericarp of Bengal coffee (16 compounds), and the leaf extract of Liberian coffee (16 compounds) (Table 11, App/Table 12).

Table 11. Compounds detected in the studied parts of coffees by HPLC-ESI-MS/MS

No.	***Proposed components***	**t_R (min)**	***CB MS***	***CA MS***	***CL MS***	***CB MP***	***CA MP***	***CL MP***	***CB IS***	***CA IS***	***CL IS***	***CB IP***	***CA IP***	***CL IP***	***CB L***	***CA L***	***CL L***
1	isocitric acid	2.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	caffeoyl hexoside	2.3		+			+		+	+							
3	quinic acid derivate	2.3					+								+		
4	citric acid	3.0			+												
5	3-caffeoylquinic acid	10.2		+	+	+	+		+	+	+		+	+	+	+	+
6	procyanidin dimer	10.2											+				
7	4-caffeoylquinic acid *	11.6			+								+			+	
8	5-caffeoylquinic acidderivative	12.1			+	+				+	+						
9	catechin/ epicatechin	12.1				+							+	+	+		+
10	procyanidin trimer	12.4													+	+	+
11	procyanidin dimer	12.7				+					+			+			+
12	5-caffeoylquinic acid *	13.1	+	+	+	+	+	+	+	+	+	+	+	+	+		+
13	4-caffeoylquinic acid *	13.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	procyanidin trimer	14.2													+	+	+
15	catechin/ epicatechin	14.7	+			+	+	+	+		+	+	+	+	+		+
16	5-caffeoylquinic acid *	14.9	+	+	+	+	+			+	+		+	+			+
17	5-coumaroylquinic acid	15.4		+	+	+	+			+	+		+				+
18	mangiferin	15.8	+			+	+						+	+	+	+	+
19	4-feruloylquinic acid	15.9	+	+	+	+	+		+	+	+		+	+			
20	5-caffeoylquinic acidmethylether	16.8			+						+						
21	5-feruloylquinic acid	17.4	+							+	+		+				

No.	*Proposed components*	t_R (min)	*CB MS*	*CA MS*	*CL MS*	*CB MP*	*CA MP*	*CL MP*	*CB IS*	*CA IS*	*CL IS*	*CB IP*	*CA IP*	*CL IP*	*CB L*	*CA L*	*CL L*
22	3,4-dicaffeoylquinic acid	18.2	+	+	+	+	+		+	+	+		+	+	+	+	+
23	3,5-dicaffeoylquinic acid	18.4			+	+	+		+	+			+	+	+	+	+
24	4,5-dicaffeoylquinic acid	19.7	+	+	+	+	+	+	+	+	+		+	+	+	+	+
25	ferulic acid	20.3	+		+	+	+		+	+	+		+		+	+	+

Abbrevations: CB: *C. benghalensis*, CA: *C. arabica*, CL: *C. liberica*, MS: mature seed, MP: mature pericarp, IS: immature seed, IP: immature pericarp, L: leaf. *: isomer of chlorogenic acid

Coffee leaf extracts

3-CQA was present in all leaf samples, being the main compound of *C. arabica* extract (App/Table 7). 5-CQA was the main component of *C. benghalensis* and *C. liberica*. 5-coumaroylquinic acid (5-CoQA) and a further isomer of 5-CQA were present in Liberian coffee. 4-CQA, diCQAs and ferulic acid, as well as mangiferin were detected in all samples. The identification of mangiferin was in concordance with earlier works (TREVISAN et al. 2016, CONÉJÉRO et al. 2014). In addition, TREVISAN et al. (2016) denoted higher total mangiferin content in the leaf of plants growing under natural full-sun conditions compared to other ones living in management used organic treatment, which was not studied in our work.

In contrast with an earlier finding (CONÉJÉRO et al 2014), we did not identified 5-caffeoylquinic acid in *C. arabica*, but procyanidin trimers were described in each leaf sample.

The results underline the presence of chlorogenic acids detected in our previous LC/MS studies which analysed their quantity in the non-hydrolysed extracts of the leaf and the immature fruit in Bengal coffee (Chapter 4.2.1.).

Immature pericarp extracts

5-CQA was characterized as the main component of all samples, additionally, 4-CQA and catechin/epicatechin also were abundant in all studied species (App/Table 8). Moreover, Arabic and Liberian coffee contained 3-CQA, a further isomer of 5-CQA, 4-feruloylquinic acid (4-FQA), 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, as well. Our actually findings are in concordance with our previous studies as ferulic acid was identified in both investigations. Even though, according to our previous studies, the quantified ferulic acid concentration was insignificant in the immature pericarp of Bengal coffee (Chapter 4.2.1.).

Immature seed extracts

The main compound of all coffee immature seed samples was 5-CQA, followed by 4-FQA, 4-CQA and 3-CQA (Table 11). 5-CQA, 5-CoQA and 5-FQA were identified in Arabic and Liberian coffees. The latter was characterized by the presence of a procyanidin dimer and 5-CQA methyl ether that could be detected only in both immature and mature seed of Liberian coffee. The diCQA compounds were described for *C. benghalensis* and *C. arabica*, while Liberian coffee contained only 3,4-diCQA and 4,5-diCQA (App/Table 9).

According to our previous studies, the ferulic acid concentration was the same in the non-hydrolysed extract made of the immature seed than in the leaf of Bengal coffee. The results overlap with the findings of an earlier comprehensive work (FARAH and DONANGELO 2006) who also mentioned the presence of chlorogenic acids. Even though they also described these phenolic compounds in the immature seed of Arabic coffee, we identified in plus isocitric acid, caffeoyl hexoside, and catechin/epicatechin. However, they mentioned in their review the presence of 5-caffeoylquinic acid, this compound was not identified in our green seed sample of Arabic coffee.

The results overlap with our earlier work as the quantity of chlorogenic acids were three times higher in the non-hydrolysed extract made of the immature seed of Arabic coffee than that of Bengal coffee (Chapter 4.2.1.).

Mature pericarp extracts

Similarly to the immature seed samples, the main compound of the mature pericarp extracts was 5-CQA, while 4-CQA was present in smaller amount in each species. For Bengal and Arabic coffees, 3-CQA, 5-CQA, 5-CoQA, 4-FQA and diCQA compounds were described, as well. Liberian coffee extract contained only 4,5-diCQA. Flavan-3-ols and a procyanidin compound were detected in Bengal coffee (App/Table 10).

Mature seed extracts

In the mature seed extracts, 5-CQA was identified as the main compound in Bengal and Arabic coffee, while 4-CQA, 5-CQA,4-FQA and the diCQAs were detected as minor components (App/Table 11). In addition, Bengal coffee was characterized by the presence of 5-FQA. The 5-CQA and 4-CQA isomers were detected with comparably high intensity in Liberian coffee extracts. Minor components of the mature seed samples were 3-CQA, 5-CoQA, 4-FQA and diCQAs. Only the sample of *C. liberica* contained citric acid. KY et al. (2007) and CAMPA et al. (2005) also identified and quantified chlorogenic acids in the fruit of Liberian coffee, which results confirm our more detailed studies. Catechin, epicatechin, flavonols, anthocyanidins, flavan-3-ols and hydroxycinnamic acids like caffeoylquinic acid, its derivatives and *p*-coumaroylquinic acid studied by ASCENSION et al. (2004) support our investigations and underline that the constitutive units of Arabic coffee fruit were mainly epicatechin, representing more than 90% of the proanthocyanidin units.

4.2.3. Total polyphenol and tannin content

The highest tannin content was found in the leaf of Arabic coffee followed by the immature pericarp of Bengal coffee and the leaf of Liberian coffee (Table 12). The least tannin content was detected in the mature seed extract of each species and in the immature pericarp of Liberian coffee (Fig. 24).

The highest polyphenol content was measured in the leaf of each species while the least content was observed in the mature pericarp of *C. liberica*. In addition, a high polyphenol concentration value was detected in the mature seed of all three species (Fig. 25).

The leaf of Arabic coffee had the highest total tannin, polyphenol content compared to all parts of the studied species (PATAY et al 2016b).

In relation to the seed extracts, the total polyphenol content of immature seed of Bengal coffee were lower than its tannin content (PATAY et al. 2016b). Our present results overlap with our previous work as the total tannin content of mature seed extract of Bengal coffee were insignificant in comparison with the other plant parts. However, the scavenger activity of mature seed extract of Liberian coffee was the highest, the total polyphenol content of mature seeds of Bengal coffee was higher than the mature seeds of Arabic and Liberian coffee (PATAY et al. 2016b). In comparison with earlier studies, the total polyphenol content and the antioxidant activity of green seed extract of *C. arabica* were higher prepared with isopropanol and water (60:40) than in our study. These differences could be explained by different extraction methods (NAIDU et al. 2008).

In the pericarp, even though the total tannin content of the mature pericarp extract of Arabic coffee were higher than that of Bengal coffee, the total polyphenol content of the mature pericarp of Bengal coffee was the highest in comparison with the other species (PATAY et al. 2016b).

Table 12. Total tannin, polyphenol and phenolic content of the investigated plant extracts

Investigated coffees and their parts		Total tannin content (%)	Total polyphenol content (%)
C. arabica	mature seed	**0.558**	2.123
	immature seed	1.084	4.146
	mature pericarp	0.793	1.687
	immature pericarp	0.838	**1.634**
	leaf	**3.498**	**7.812**
C. benghalensis	mature seed	**0.447**	3.285
	immature seed	0.581	2.503
	mature pericarp	0.745	**2.235**
	immature pericarp	**1.464**	3.677
	leaf	1.195	**6.258**
C. liberica	mature seed	0.402	3.129
	immature seed	1.164	3.120
	mature pericarp	1.134	**0.938**
	immature pericarp	**0.344**	1.863
	leaf	**1.341**	**5.387**

Coloured values: green: lowest value, blue: highest value among the samples used the same method.

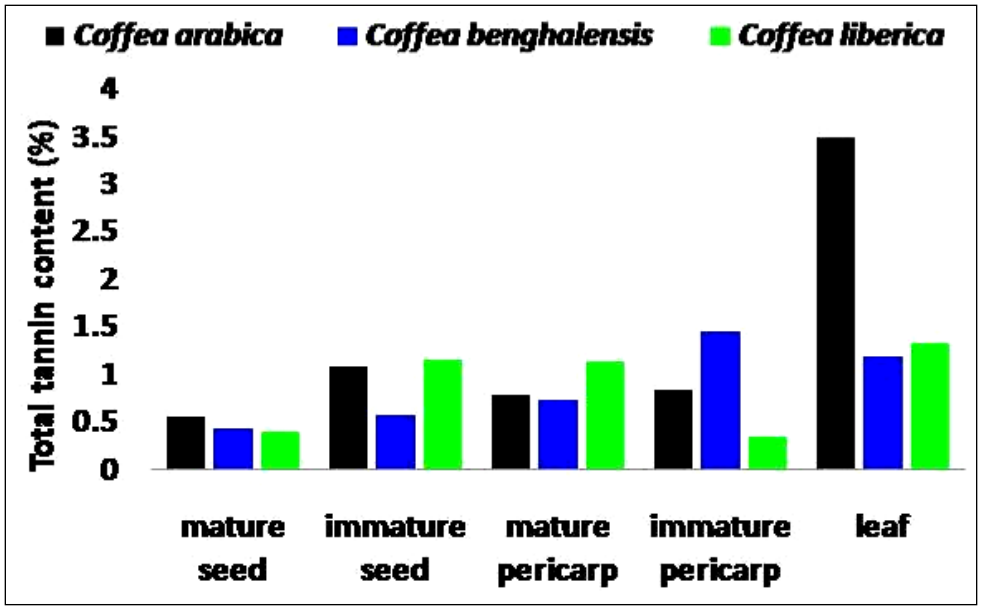

Fig. 24. Total tannin content of the studied *Coffea* species measured by spectroscopic method

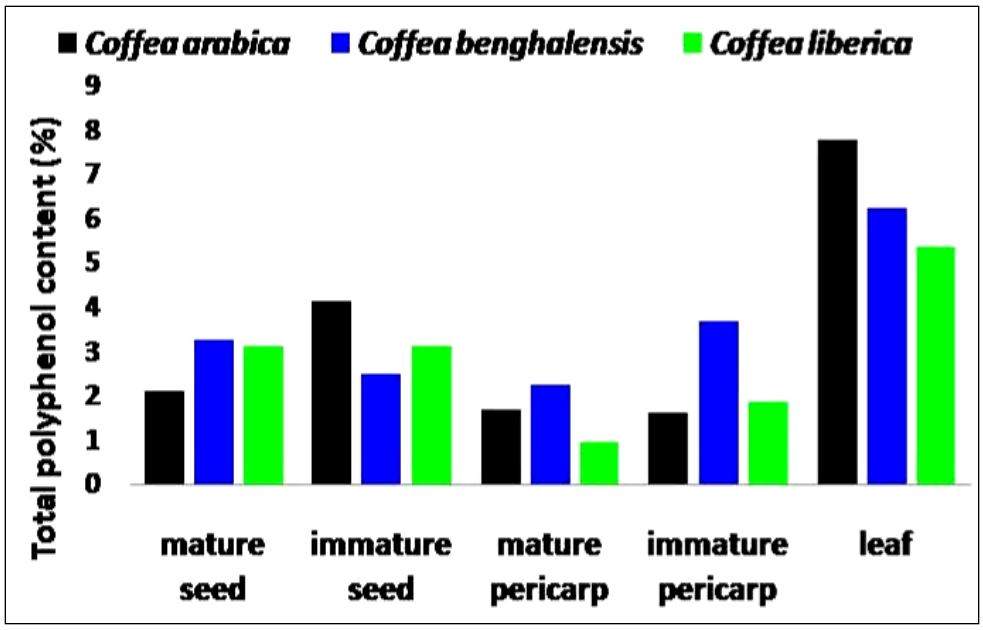

Fig. 25. Total polyphenol content of the studied *Coffea* species measured by spectroscopic method

4.2.4. Conclusions

The 31 identified phenolic compounds of the studied species suggest a significant chemical diversity of coffees which have a great impact in chemotaxonomical aspect, as well.

The leaf of Bengal, Arabic and Liberian coffee produced a high phenolic content. The immature pericarp of Bengal coffee showed a significant tannin, polyphenol and phenolic contents similar to Arabic samples, in addition, the measured polyphenol value of the mature pericarp and seed of Bengal coffee were also high compared to the other samples.

Our findings provide relevant new information about a less studied wild Bengal coffee that may be of similar significance as Arabic and Liberian coffee. We could summarise that our findings completed the scientific literature data of Bengal coffee which can present new opportunities and challenges for further phytochemical and medical studies of the species.

5. Antioxidant activity of the selected *Coffea* species

Antioxidants are capable to delay or inhibit the oxidation processes. These take place under the influence of atmospheric oxygen or reactive oxygen species. Due to this reason, antioxidants are used for the stabilization of polymeric products of petrochemicals, foods, cosmetics, and pharmaceuticals. In addition, they are involved in the defense mechanism of the organisms against pathologies associated to the attack of free radicals. There are endogenous antioxidants like enzymes and exogenous synthetic and natural antioxidants like vitamins, flavonoids, anthocyanins, and some mineral compounds etc. There are numerous known antioxidant compounds in plants and beverages, e.g. carotenoids, phenolic compounds, benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans, and lignins (DURAK et al. 2014, RAMALAKSHMI et al. 2008, PISOSCHI and NEGULESCU 2011).

Special phytochemical and analitycal methods can be used to describe and quantify antioxidants and their significant effect on the human body. Spectrometric (ECL, ORAC, DPPH, Folin-Ciocalteu, total polyphenol and tannin content, ABTS, FRAP, PFRAP, CUPRAC, HORAC, TRAP, fluorimetry), electroanalytical (cyclic voltammetry, amperometry, biamperometry), and chromatography methods (gas and high performance liquid chromatography) are able to offer a complete profile of the antioxidant content of foods (PISOSCHI and NEGULESCU 2011). Among our used methods, ORAC and the ECL assays belong to the hydrogen atom transfer (HAT) mechanism group while the DPPH technique is considered to be based on single electron transfer (SET) (PRIOR et al. 2005). Both techniques are considered to characterize the non-enzymatic total antioxidant capacity of plant extracts. ECL method applying phenolic compound as enhancer proved to be more sensitive than ORAC assay, however, the measuring range and precision of ORAC method were more favorable. In ECL technique, phenolic enhancer compound itself is also involved in the reaction with POD intermediates accelerating the reaction by increasing the turnover rate of the enzyme. Apart from POD-phenolic interaction the resulting phenoxyl radicals can directly oxidize luminol (EASTON et al. 1996). It is uncertain yet why the TE/g values obtained by ORAC HAT assay were considerably higher than those seen for the other two methods. It might be postulated that in the ORAC microenvironment more antioxidant compounds could react with the AAPH oxidant than in DPPH (SET) and ECL (HAT) assays.

The aim of our antioxidant experiments was to confirm the antioxidant activity of the extracts of *C. benghalensis,* and to compare the results with those of the well know *C. arabica* and *C.*

liberica. Nowadays, science is focused on exogenous and natural antioxidants of plants like polyphenols, because they are considered to be safer, more environmental friendly and having less secondary effects than synthetic compounds. Due to this reason these analyses were carried out to find new sources of natural antioxidants for nutraceuticals, and a new utilization of wasted residues of coffee products. The previously published antioxidant techniques used to thr studied species were complemented with our techniques in Table 13.

Table 13. Summary of the used methods of earlier papers and of our work related to the antioxidant activity of the studied coffees

Studied species and their parts		***ECL***	***DPPH***	***ORAC***	***FRAP***	***CUPRAC***	***ABTS$^+$***	***Ranc***
	mature s.	+	+ [1]	+	[1]	-	[3]	[4]
	immature s.	+	+ [5]	+	-	[2]	-	-
C. arabica	mature p.	+	+ [6]	+	-	-	[6]	-
	immature p.	+	+	+	-	-	-	-
	leaf	-	-	-	-	-	-	-
	mature s.	+	+ [7]	+	-	-	-	-
C. bengha-lensis	immature s.	+	+	+	-	-	-	-
	mature p.	+	+ [7]	+	-	-	-	-
	immature p.	+	+	+	-	-	-	-
	leaf	-	-	-	-	-	-	-
	mature s.	+	+	+	-	-	-	-
	immature s.	+	+ [8]	+	[8]	-	-	-
C. liberica	mature p.	+	+	+	-	-	-	-
	immature p.	+	+	+	-	-	-	-
	leaf	-	-	-	-	-	-	-

Abbreviations: mature s.: mature seed, immature s.: immature seed, mature p.: mature pericarp, immature p.: immature pericarp, ECL: Enhanced chemiluminescence method, DPPH: 2,2-diphenyl-1-picrylhydrazyl method, ORAC: Oxygen Radical Absorbance Capacity assays, FRAP: Feric Reducing power assay (Fe^{3+} and Fe^{2+}), CUPRAC: Cupric ion reducing antioxidant capacity, $ABTS^+$: radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid), Ranc: Rancimat assay, + our test

References: [1] ANKALABASAPPA et al. 2014; [2] SKOWRON et al. 2016; [3] HERNÁNDEZ et al. 2012; [4] GEBEYEHU and BIKILA 2015; [5] BABOVA et al. 2016; [6] DUANGJAI et al. 2016; [7] KIRAN et al. 2011; [8] CHEE et al. 2015

5. 1. Materials and methods

The studies on the antioxidant potential of the selected coffees were carried out at the Department of Laboratory Medicine, Medical School, University of Pécs.

5.1.1. Chemicals

All chemicals, used for antioxidant assays, were of analytical or spectroscopic grade purity and highly purified water (<1 μS) was used in our experiments. Horseradish peroxidase (POD from Sigma-Aldrich), 1 mg/mL bovine serum albumin (BSA, Serva) in 50 mM phosphate buffer pH 7.4, H_2O_2 (Molar Chemicals Ltd.) diluted with citric acid (Ph.Hg. Eur 7th), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), luminol, para-iodophenol, diphenyl-2-picryl-hydrazyl (DPPH stable free radical), fluorescein-Na_2 salt, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH, all from Sigma-Aldrich), methanol and ethanol (Reanal, Hungary) were used as received. In the ORAC assay, 75 mM phosphate buffer of pH 7.4 was applied.

5.1.2. Sample preparation

The plant samples of *C. arabica, C. benghalensis* and *C. liberica* were collected in the Botanical Garden, University of Pécs in the spring of 2014 and 2015. Voucher specimens of each species were deposited and labelled with unique codes at the Department of Pharmacognosy, University of Pécs. Samples were collected from 2 plants per each species.

For all used antioxidant assays (ECL, DPPH, ORAC), the immature and mature pericarp and seed of each species were ground (0.25 g each) and extracted with 5 mL 50% ethanol. The extracts were shaken for 20 min (KL-2, Edmund Bühler GmbH, Germany), then they were filtered and stored at 4^{o}C in the dark until analyses (less than 7 days). We could not study leaf samples as they contain chlorophyll and for this reason the solutions were not clear.

5.1.3. 2,2-diphenyl-1-picrylhydrazyl method (DPPH)

4 mg DPPH was prepared in 100 mL methanol (0.1 mmol/L) and kept in the fridge being stable for at least 1 week. For absorbance measurements standard 96-well microplates (Sarstedt) were applied. 20 μL Trolox/blank/sample and 180 μL DPPH solution were pipetted into the wells (using a multichannel pipette), then they were mixed, and the absorbance was read at 517 nm after 30 minutes incubation in the dark at 25 °C (CHEN et al. 2013, JIMÉNEZ et al. 2014, SHARMA and BHAT 2009, VIGNOLI et al. 2011).

5.1.4. Enhanced chemiluminescence method (ECL)

We adapted and modified the method of Muller et al. (MULLER et al. 2013) as follows.

Reagents: Before the analysis 15 μU/mL POD working solution was freshly prepared from 1.5 U/mL POD stock stored at -20 °C in phosphate buffered saline (PBS, pH 7.4) by dilution with the BSA containing phosphate buffer and was kept on ice. A working reagent of 1360 μM H_2O_2 was also freshly diluted with 0.1% citric acid from 10 M concentrated stock solution and was also kept on ice, protected from light. During the whole period of measurements these reagents were stored in melting ice. Both working solutions were stable for at least several hours.

The chemiluminescence detection reagent was prepared separately by dissolving luminol and *p*-iodophenol in 0.2 M boric acid/NaOH buffer, pH 9.6 and was refrigerated at 4 °C in brown bottles with a shelf life of several weeks. Trolox was used as standard in both assays. Trolox at 1 mM concentration was dissolved freshly in 50% ethanol weekly and kept at 4 °C. Depending on the assay, Trolox dilutions in the range of 0-100 μM were prepared on the day of the experiments with the same diluents applied for the samples.

ECL antioxidant method: The chemiluminescence reaction was performed in 96-well white optical plates (Perkin-Elmer). The enzyme working solution and the ECL reagent was premixed (200 μL POD + 70 μL ECL reagent) and kept on ice. The wells were filled with 20 μL Trolox/blank/sample and 270 μL of POD-ECL reagent was pipetted into each well with an 8-channel micropipette. The reaction was initiated by automated injection of 20 μL ice-cold H_2O_2 in citric acid (final concentrations of the components in the wells: 0.97 μU/mL POD, 101.6 μM luminol, 406.4 μM *p*-iodophenol, 88 μM H_2O_2). The chemiluminescence signal was followed for 20 min.

5.1.5. Oxygen radical absorbance capacity assays (ORAC)

4 μM fluorescein (FL) stock was prepared in 75 mM phosphate buffer of pH 7.4 (stable for 1 week in the fridge). The working FL solution was made freshly diluting the stock with phosphate buffer at a 1:99 ratio (40 nmol/L). AAPH was also prepared just before the measurements in phosphate buffer (400 mM). Trolox standards were used as described above. Into each well of black optical plates (Perkin Elmer) 25 μL of blank/standard/sample and 150 μL of diluted FL were pipetted and the plates were preheated to 37 °C for 20 min. The outer wells of the plates were filled with 200 μL phosphate buffer, and only the inner 6x10 matrix was used for the assay.

The reaction was initiated by automated injection of 25 µL AAPH solution into each well, and fluorescence intensities were immediately monitored for 80 min (490/520 nm) at 150 s intervals. The final concentrations of the components in the wells were as follows: FL 30 nM, AAPH 50 mM, Trolox 0 – 33.3 µM (DÁVALOS et al. 2004, GILLESPIE 2007).

5.1.6. Instrumentation and interpretation of data of antioxidant tests

For the ECL based measurements a Biotek Synergy HT plate reader equipped with programmable injectors was used.

After initiation of the reaction by injection of H_2O_2, light detection was immediately begun with 0.2 s measuring time/well for 20 min at 64 s measuring intervals. Trolox standards in 50% ethanol were applied in the range of 0-15 µM final concentrations in the wells and a 32-fold dilution with 50% ethanol of the plant extracts were used for the measurements (n = 12 replicates for each sample). The total antioxidant capacity (TAC) of the extracts was calculated from the regression equation obtained for the standards, multiplied by the dilution factor and expressed as µM Trolox equivalent (TE). TE for each plant extract was referred to 1 g of initial dry material.

For the DPPH assay, a Perkin Elmer EnSpire Multimode reader was used in absorbance mode, equipped with monochromators. Standardization of the assay was done by application of 0-25 µM Trolox/well final concentrations in 50% ethanol, and absorbance values were read at 517 nm after 30 min of incubation at 25 °C (with 5 s shaking before the measurement). Antioxidant capacities were calculated either by using the equation of the calibration line or by expressing the antioxidant activity of the extracts in % of the blank using the formula: $(A_{blank} – A_{sample}/A_{blank})*100$ (LU et al. 2014). TAC values were also referred to 1 g of dried plant and were given as TE/g or % TAC/g.

For the ORAC assay the Biotek Synergy HT plate reader was used in fluorescence mode at 37°C with 490 nm excitation and 520 nm emission filter settings. After 20 min incubation of the plate containing blanks/standards/samples and FL at 37°C the AAPH start reagent was automatically injected into the wells and readings were taken in every 150 s for 80 min with 100 intensity readings/well at each measuring point. TE was calculated by subtraction of the fluorescence intensities of the corresponding blank values from those of the Trolox standards (net area under curve, nAUC) and in this way a calibration line was obtained based on nAUC vs. Trolox concentrations. TE data for the examined plants were obtained from the regression equation of the standards and they were also referred to 1g of dry plant.

5. 2. Results and discussion

5.2.1. Results of the applied antioxidant tests

We could quantify the antioxidant activity by all three methods in each tested plant extract. The ECL and DPPH TE/g values showed loose correlation (R^2=0.587, p=0.083 by Student's t-probe) while those obtained for the ORAC assay were considerably higher with a more uniform pattern and without correlation with the other two assays' data (Table 14). The imprecision of the three assays was acceptable (ECL: =<5%, DPPH: =<10%, ORAC: =<2%). The DPPH data were also calculated as % TAC using the equation described in 2.8. Our results showed closer correlation between the ECL method and the percentage antioxidant capacity obtained by the DPPH technique (R^2=0.6107, p=0.161 by Student's t-probe). The biggest difference was found in the immature pericarp of *C. benghalensis* and the mature pericarp of *C. liberica* where the DPPH method showed much higher antioxidant capacity than the ECL assay (Fig. 26,27). In our experiments, ORAC technique showed the highest values which did not correlate with the results of the other two assays of each sample. In contrast to the data of KIRAN et al. (2011), we found lower antioxidant activity in the case of the mature fruit extract of *C. benghalensis*, while the value of the immature pericarp was higher in our study measured by DPPH method. For the reason of low R^2 value the different applied methods can be mentioned which diverse radicals and diverse neutralization result. In addition, the detector system and the antioxidants also differ in the assays.

The scavenger activity of the mature pericarp of Liberian coffee was the most significant among the studied plants (PATAY et al. 2016b). Although *C. liberia* is less used in trade, the antioxidant effect of its green seeds is comparable to that of *C. arabica* and *C. robusta* (TEO et al. 2014). In order to explain the differences between the various methods, we plan to separate the active compounds of *Coffea* species and measure their antioxidant capacity separately by all techniques.

Table 14. Total antioxidant capacity of the selected *Coffea* species measured by three different spectroscopic methods

Investigated species and their parts		DPPH assay	Chemiluminescence assay	ORAC assay
		TE (µmol/g dried plant) ± SD	TE (µmol/g dried plant) ± SD	TE (µmol/g dried plant) ± SD
C. arabica	mature seed	1627.089 ± 158.675	1628.254 ± 20.700	5139.01 ± 16.01
	immature seed	1019.291 ± 94.423	1793.257 ± 1.332	5914.72 ± 78.76
	mature pericarp	1429.857 ± 119.541	1570.281 ± 28.951	4816.47 ± 66.83
	immature pericarp	1205.598 ± 73.270	1347.583 ± 35.621	4327.14 ± 39.41
C. benghalensis	mature seed	1691.492 ± 153.326	1773.039 ± 34.323	5640.41 ± 68.91
	immature seed	1598.913 ± 139.436	1791.784 ± 1.662	5608.26 ± 23.26
	mature pericarp	1702.417 ± 146.458	1815.860 ± 28.173	5501.75 ± 51.16
	immature pericarp	3132.134 ± 121.553	1862.025 ± 166.608	5558.02 ± 35.86
C. liberica	mature seed	2212.817 ± 204.156	2745.598 ± 74.097	5872.52 ± 52.23
	immature seed	1200.423 ± 106.076	1750.142 ± 9.326	5415.33 ± 15.04
	mature pericarp	3307.812 ± 93.589	3386.733 ± 40.773	2740.56 ± 38.68
	immature pericarp	2070.049 ± 159.152	2396.324 ± 145.552	5721.80 ± 8.53

Abbreviations: TE: Trolox equivalent in µM. Data are referred to 1 g of dried plant material, N=12 for the ECL and DPPH methods respectively, while in the ORAC assay measurements were performed in triplicates.

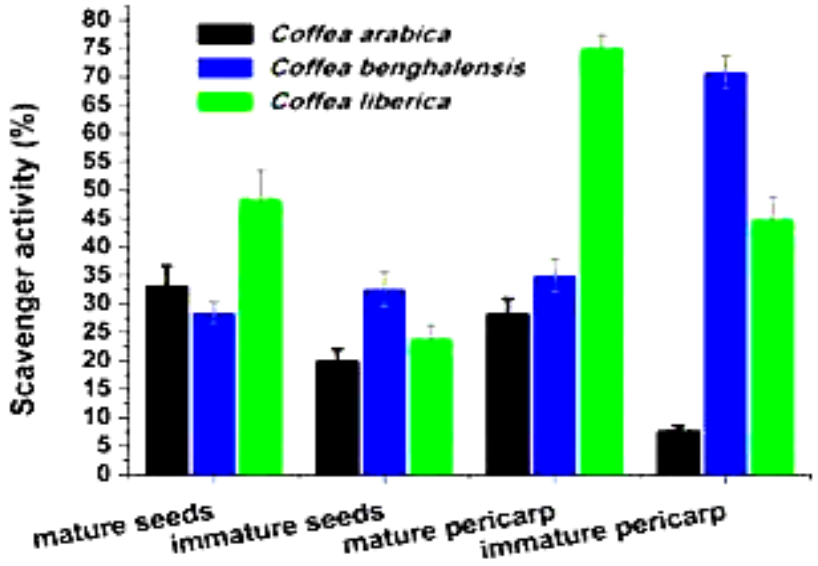

Fig. 26. Scavenger activity of the studied *Coffea* species measured by DPPH method

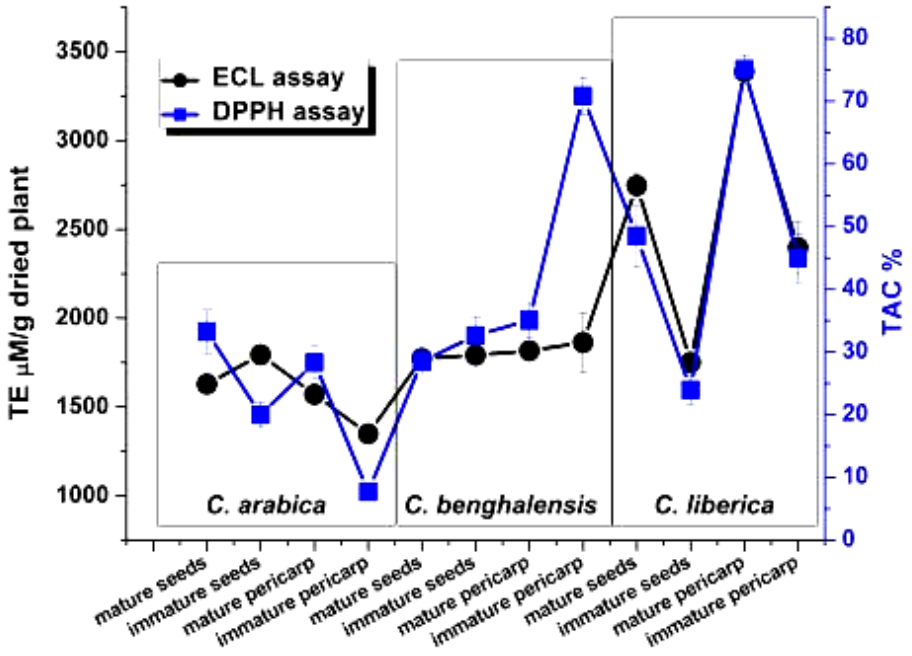

Fig. 27. Comparison of the results by ECL and DPPH assays. Data obtained from the extraxts of the studied coffees are expressed in TE/g dried plant (ECL method) and in TAC % (DPPH method).

5.2.2. Conclusions

In the case of Bengal coffee, highest values were detected in the immature seed and pericarp used DPPH, and the mature pericarp by ORAC method compared to the other studied species. The values measured by ECL assay were the highest in each studied part of Liberian coffee.

Among the used antioxidant assays, the measured ECL and DPPH values indicated a loose correlation in contrast with the data obtained by ORAC assay, while a closer correlation was observed between the ECL technique and the expressed antioxidant potential studied by the DPPH method in the samples of each coffee species. The much higher antioxidant activities measured by ORAC assay might reflect the differences in the reactive antioxidant compounds among the assays and/or the altered reactivity with the reporter molecules.

6. Antimicrobial activity of the selected *Coffea* species

In recent years, multiple drug resistance in both human and plant pathogens has developed due to the indiscriminate use of commercial antimicrobial drugs commonly applied in the treatment of infectious diseases. Nowadays medicinal plants are becoming the centre of attention instead of chemical medicines as they have less secondary actions. Due to this reason the traditional medicines remain the most affordable, and easily accessible source of treatment for many communities around the world.

Since bacterial infections represent a serious therapeutic problem nowadays, the purpose of this study was to investigate the antimicrobial effect of *C. arabica, C. benghalensis* and *C. liberica.* The analysis were carried out to find new sources of natural nutraceuticals with antimicrobial effect, and a new utilization of wasted residues of coffee products too. Diffusion methods are often used to study antibacterial activity of plant extracts. In our work, disc and agar diffusion methods were selected to examine the antibacterial activity of the selected *Coffea* species. The activity of the extracts was estimated according to the diameter of the inhibition zones.

The selected strains, namely *Methicillin-resistant Staphylococcus aureus* (*MRSA), Staphylococcus aureus, Bacillus subtilis* and *Streptococcus agalactie,* are all Gram-positive bacteria which can cause different infections in the human body. *Staphylococcus aureus* can be found on the normal human skin flora and mucous membranes and it can infect people in the case of skin or mucous membrane injuries. *Methicillin-resistant Staphylococcus aureus (MRSA)* is responsible for 25-60% of the severe nosocomial infections and it has developed resistance to β-lactam antibiotics. In addition, this resistance can be coupled with other resistances resulting in multidrug resistance. *Bacillus subtilis* can be found in the soil and in the human intestines which can cause food poisoning in large quantities (GERGELY 2003). *Streptococcus agalactie* can cause serious problems mostly in newborns (e.g pneumonia), while it causes rarely infections in adults (PAL 2013).

Some previously published antimicrobial studies of *Coffea* species complemented with our investigations are described in Table 15.

Table 15. Summary of the tested bacteria in earlier papers and in our work related to the antimicrobial activity of the studied coffees

	C. arabica					***C. benghalensis***					***C. liberica***				
	leaf	ip	mp	is	ms	leaf	ip	mp	is	ms	leaf	ip	mp	is	ms
E. coli					[2]			[1]		[1]					
P. vulgaris								[1]		[1]					
P. mirabilis					[2]										
P. hauseri					[2]										
S. typhimurium					[4]			[1]		[1]					
S. enterica					[2]										
S. typhi					[3,4]										
K. pneumoniae								[1]		[1]					
K. oxytoca					[2]										
S. faecalis								[1]		[1]					
S. aureus	+	+	+	+	+[3]	+	+	+[1]	+	+[1]	+	+	+	+	+
MRSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. agalactie	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. freundii					[2]										
S. marcescens					[2]										
E. aerogenes					[2]										
E. cloacae					[2]										
E. faecalis					[3]										
P. aeruginosa					[3]										
V. cholerae					[4]										
S. flexneri					[3]										
S. epidermidis					[3]										

Abbreviations: ip: immature pericarp, mp: mature pericarp, is: immature seed, ms: mature seed, + our test

Bacteria strains: *E. coli: Escherichia coli, P. vulgaris: Proteus vulgaris, P. mirabilis: Proteus mirabilis, P. hauseri: Proteus hauseri, S. typhimurium: Salmonella typhimurium, S. enterica: Salmonella enterica, S. typhi: Salmonella typhi, K. pneumonia: Klebsiella pneumoniae, K. oxytoca: Klebsiella oxytoca, S. faecalis: Streptococcus faecalis, S. aureus: Staphylococcus aureus, MRSA: Methicillin-resistant Staphylococcus aureus, B. subtilis: Bacillus subtilis, S. agalactie: Streptococcus agalactie, C. freundii: Citrobacter freundii, S. marcescens: Serratia marcescens, E. aerogenes: Enterobacter aerogenes, E. cloacae: Enterobacter cloacae, E. faecalis: Enterobacter faecalis, P. aeruginosa: Pseudomonas aeruginosa, V. cholerae: Vibrio cholerae, S. flexneri: Shigella flexneri, S. epidermidis: Staphylococcus epidermidis*

References: [1] KIRAN et al. 2011; [2] ALMEIDA et al. 2006 [3] ARORA et al. 2009; [4] SHETTY et al. 1994

6. 1. Materials and methods

The antimicrobial investigations were performed at the Department of Medical Microbiology and Immunology, Medical School, University of Pécs.

6.1.1. Chemicals

Chemicals used for microbiological studies were the followings: 50% ethanol (Merck), Müller-Hinton Agar (Oxoid, UK), distilled water, NaCl 0.9% (Molar Chemicals Ltd.), and vancomycin (Bio Rad).

6.1.2. Sample preparation

The plant samples of *C. arabica, C. benghalensis* and *C. liberica* were collected in the Botanical Garden, University of Pécs in the spring of 2014 and 2015. Voucher specimens of each species were deposited and labelled with unique codes at the Department of Pharmacognosy, University of Pécs. Samples were collected from 2 plants per each species.

The leaf, immature and mature seed and pericarp (0.5 g) were powdered and extracted with 5 mL of 50% ethanol. The solutions were heated on ultrasound water-bath (Elmasonic S 30, Simex) for 20 min at 40°C, and then they were filtered (PHARMACOPOEA HUNGARICA 8th ed. 2006).

6.1.3. Disc diffusion and agar diffusion methods

During the substrate preparation, a mixture of 1 L of distilled water and 38 g of Mueller-Hinton agar was boiled for 30 min, and then 25-25 mL of it was poured into Petri dishes.

For disc diffusion method, 48 discs were kept in refrigerator until the solution was frozen. The bacteria were suspended in 1.5 mL NaCl 0.9%, then they were separately applied onto the substrate. After this, filter paper discs were placed onto the substrate with 5 mm diameter and 10-10 uL of the tested extracts and standard solution were applied onto the discs.

For agar diffusion method, 100 μL of extracts were measured into the holes made in the agar using sterile metal borers.

The leaf, seed and pericarp extracts were tested by disc diffusion, while those of the leaves also by agar diffusion method against four Gram-positive bacteria like *MRSA* (isolated from a haemoculture of a septic patient), *S. aureus* (ATCC 29213), *B. subtilis* (ATCC 6633), and *S.*

agalactie strains (isolated from a vaginal excretion). *S. aureus* and *B. subtilis* were taken from Hungarian collections, while *MRSA* and *S. agalactie* were isolated at a local hospital. As standard antibiotic 5 μg/mL vancomycin was used. The 48 discs were incubated at 37°C for 24 h. The inhibition zones were measured in mm. The tests were carried out in triplicate (SUHANYA et al. 2009, KIRAN et al. 2011, KIRAN et al. 2012, FAWOLE et al. 2013).

6. 2. Results and discussion

6.2.1. Disc diffusion method

The antibacterial activity (inhibitory zone) of the studied parts of the selected *Coffea* species were compared with commercial antibiotic 5μg/mL vancomycin (n = 3) (Table 16).

However, Arabic and Liberian coffee were less effective than Bengal coffee, many reports described their positive antimicrobial activity against various strains (DAGLIA et al. 1994, SHETTY et al. 1994, ALMEIDA et al. 2006).

In comparison, the leaf of Arabic coffee had the highest while the mature seed of Liberian coffee the lowest inhibition against the tested strains. The inhibitory zones of the extracts of Bengal coffee were mostly wider for each strain than those of the other two species. The lowest antimicrobial activity is concerned to the immature pericarp and mature seed of Liberian coffee, as well as the mature seed of Bengal coffee which showed insignificant inhibition for *S. agalactie* and *B. subtilis.* Our results are in concordance with earlier data about antibacterial properties of the fruit of Bengal coffee tested against *P. vulgaris*, *E. coli*, *K. pneumoniae*, *S. typhimurium*, *S. faecalis* and *S. aureus*, which we completed with the detected inhibitory effect of the leaf and the immature fruit (KIRAN et al. 2011, 2012).

The strongest inhibitory effect against *MRSA* was observed in the leaf of Arabic coffee, while the less one was found in the mature pericarp of Bengal coffee. Against *B. subtilis*, which was most sensible bacteria, showed bigger inhibitory zones in all extracts, the most significant effect had the leaves of Arabic and Bengal coffee. In the case of *S. agalactie*, the most inhibitory activity was described in the leaf extract of Arabic coffee, but the less one in the immature pericarp of Liberian coffee.

Table 16. Antimicrobial activity of the studied *Coffea* species used disc diffusion method

Investigated species and their used parts		Inhibitory zone (mm) and SD (%)		
		MRSA	*Bacillus subtilis*	*Streptococcus agalactie*
*C. arabica**	leaf	15.33±0.57	**15.66±1.15**	11.66±1.30
	mature seed	-	-	-
	immature seed	-	-	-
	mature pericarp	-	-	-
	immature pericarp	9.66±0.57	**10.66±2.08**	-
*C. benghalensis**	leaf	13.66±1.15	**14.66±0.57**	8.33±1.52
	mature seed	8.66±0.57	**9±0.00**	7.33±0.57
	immature seed	-	**9.33±1.15**	9±0.00
	mature pericarp	8±0.00	**10±0.00**	9.33±1.15
	immature pericarp	10±0.00	**11.66±1.15**	8.33±1.52
*C. liberica**	leaf	12.66±1.52	**13±1.73**	10.33±2.08
	mature seed	-	**7.33±0.57**	-
	immature seed	-	-	8±0.00
	mature pericarp	-	**10±0.00**	-
	immature pericarp	11±0.00	**11.33±1.52**	6±1.14
Vancomycin 5µg/mL		22±0.00	**27±0.00**	18±0.00

*0.5 g of each sample was extracted with 5 mL of 50% ethanol, then 10 uL extraction was applied.

6.2.2. Agar diffusion method

The leaf extracts of each species were studied also by agar diffusion method. The results compared with commercial antibiotic 5µg/mL vancomycin (n = 3) are illustrated in Table 17.

The results overlap with our previous data related to the leaf extract tested by disc diffusion method. The most sensible bacteria were *MRSA* and *B. subtilis*, while *S. agalactie* admitted as the less sensible strains. In comparison, the leaf extract of *C. arabica* and *C. benghalensis* showed the strongest antibacterial effect against *MRSA* and *B. subtilis* used this test.

The antibacterial effect of the roasted seeds of Arabic coffee was also reported earlier against *B. subtilis* (DAGLIA et al. 1994). In addition, due to their chlorogenic acid content and Maillard reaction products which are generated during seed roasting, the extracts of Arabic coffee showed an inhibitory effect against e.g. *S. aureus* which was also successfully tested in our study used the leaf extract of the studied coffee species (DAGLIA et al. 1994), Chlorogenic acids which were

detected in all species by HPLC-ESI-MS/MS support earlier results related to the antibacterial effect of phenolic acids of the seed of Arabic coffee (LOU et al. 2011, DAGLIA et al. 1994).

Table 17. Antimicrobial activity of the leaves of the studied *Coffea* species used agar diffusion method

Leaf of the investigated coffee species	Inhibitory zone (mm) and SD (%)			
	MRSA	*Bacillus subtilis*	*Staphylococcus aureus*	*Streptococcus agalactie*
*C. arabica**	**15.11±0.57**	**15.30±0.53**	**12.51±1.30**	-
*C. benghalensis**	13.12±0.15	**15.30±0.53**	11.10±0.17	-
*C. liberica**	14.23±0.72	12.42±1.15	11.65±1.25	11.43±1.14
Vancomycin 5µg/mL	20±0.00	20±0.00	20±0.00	20±0.00

Bold numbers: highest values of the samples tested against the same bacteria strains.
*0.5 g of each sample was extracted with 5 mL of 50% ethanol, then 10 uL extraction was applied.

6.2.3. Conclusions

The antimicrobial activity of the leaf, the immature and the mature pericarp and seed were tested against *MRSA, B. subtilis, S. agalactie* and *S. aureus* used disc and agar diffusion methods. As conclusion, the extracts of all studied parts of Bengal coffee showed inhibitory activity against *MRSA, B. subtilis, S. agalactie* and *S. aureus.* The most significant effect had its leaf and that of Arabic coffee against *B. subtilis.* Even though, wild Bengal coffee has a less caffeine content than cultivated species, our studies confirmed its similar antibacterial activity with Arabic and Liberian coffee.

7. Summary and novel findings

The study realised a comparative histological, phytochemical and antimicrobial analysis of three *Coffea* species namely the widely known and cultivated *C. arabica* and *C. liberica*, and a less studied *C. benghalensis* as a wild species. The determined aims of the work were accomplished highlighting mostly the new records of Bengal coffee in each point as follows.

- In histological aspect, the leaf of Bengal coffee can be characterised with thicker cuticle and higher palisade cells extended in 2 rows, highest epidermis cells in the petiole and the stem, and widest and highest parenchyma cells in the fruit compared to *C. arabica* and *C. liberica.*
- In phytochemical aspect, 34 phenolic compounds were identified by HPLC in the leaf, immature and mature seed and pericarp of the selected plants which suggest a significant chemical diversity of coffees. According to the methods of Ph. Eur. 7.0, the leaf of Bengal, Arabic and Liberian coffee produced a high phenolic content. The immature pericarp of Bengal coffee showed a significant tannin, polyphenol and phenolic content similar to Arabic coffee, in addition, the polyphenol value of the mature pericarp and the seed of Bengal coffee were also high compared to the other plants.
- Among the results of the performed antioxidant assays, ORAC technique showed the highest values which did not correlate with the results of the other methods. In the case of Bengal coffee, highest values were detected in the immature seed and pericarp used DPPH, and the mature pericarp by ORAC method compared to the other studied coffee species. The values measured by ECL assay were the highest in each studied part of Liberian coffee.
- In the antimicrobial assays tested the leaf, the immature and the mature pericarp and seed by disc and agar diffusion methods, all studied parts of Bengal coffee showed inhibitory activity against *MRSA, B. subtilis, S. agalactie* and *S. aureus.* However, the most significant effect was detected in the leaf of Bengal and Arabic coffee against *B. subtilis*, our studies confirmed similar antimicrobial potential of Bengal coffee compared with Arabic and Liberian species against the studied bacteria strains.

Our findings provide relevant new records on the less studied wild Bengal coffee which can present new opportunities and challenges for further phytochemical and medical studies of the species.

8. References

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10. Appendix

10.1. Histological study of the selected *Coffea* species

Table 1. Histological data of the leaves of the selected coffees (n=20)

Histological characters of coffee leaves	*C. arabica* (µm)		*C. benghalensis* (µm)		*C. liberica* (µm)	
	Mean (µm)	±SD	Mean (µm)	±SD	Mean (µm)	±SD
adaxial epidermis h	27.18	±4.92	19.94	±2.73	26.69	±5.29
adaxial epidermis w	28.62	±5.61	25.00	±5.51	32.66	±7.91
abaxial epidermis h	26.55	±3.38	21.08	±7.34	21.12	±4.85
abaxial epidermis w	25.93	±6.58	22.21	±6.46	36.67	±11.53
palisade par h	30.00	±2.74	52.77	±9.51	27.01	±2.77
palisade par w	11.56	±1.53	13.88	±2.80	13.20	±2.79
spongy parenchyma h	31.25	±7.95	27.97	±5.15	25.25	±5.77
spongy par w	38.20	±8.93	31.15	±7.94	32.13	±8.03
adaxial cuticle th	3.57	±0.82	8.69	±2.40	5.02	±0.83
abaxial cuticle th	5.06	±2.07	8.61	±1.58	4.90	±0.69
vascular bundles w	223.22	±78.31	267.93	±20.39	153.84	±12.69

Abbreviations: h = height; w = width; th = thickness; par = parenchyma

Table 2. Histological data of the petioles of the selected coffees (n=20)

Histological characters of coffee petioles	*C. arabica* (µm)		*C. benghalensis* (µm)		*C. liberica* (µm)	
	Mean (µm)	±SD	Mean (µm)	±SD	Mean (µm)	±SD
adaxial epidermis h	18.18	±2.67	19.49	±3.89	15.09	±1.35
adaxial epidermis w	13.15	±2.58	18.39	±3.94	14.37	±2.81
abaxial epidermis h	20.95	±2.19	23.17	±3.05	17.43	±3.25
abaxial epidermis w	17.02	±3.97	20.94	±3.47	22.23	±5.70
parenchyma height	38.25	±6.38	38.28	±6.21	25.61	±5.29
parenchyma width	39.75	±10.98	32.57	±8.13	26.64	±5.83
adaxial cuticle th	3.09	±1.00	7.77	±1.93	3.85	±0.48
abaxial cuticle th	2.91	±0.48	5.03	±1.42	4.30	±1.03
vascular bundles w	240.44	±62.94	287.51	±48.47	156.89	±18.05

Abbreviations: h = height; w = width; th = thickness

Table 3. Histological data of the stems of selected coffees (n=20)

Histological characters of coffee stems	*C. arabica* (µm)		*C. benghalensis* (µm)		*C. liberica* (µm)	
	Mean (µm)	±SD	Mean (µm)	±SD	Mean (µm)	±SD
epidermis h	13.37	±2.21	16.41	±2.45	10.97	±2.33
epidermis w	15.67	±3.27	17.39	±3.43	16.57	±3.43
parenchyma h	29.15	±8.03	21.72	±1.39	43.64	±9.93
parenchyma w	42.00	±12.82	35.93	±0.69	46.21	±8.44

Abbreviations: h = height; w = width

Table 4. Histological data of the fruits of selected coffees (n=20)

Histological characters of coffee fruits	*C. arabica*		*C. benghalensis*		*C. liberica*	
	Mean (µm)	±SD	Mean (µm)	±SD	Mean (µm)	±SD
epidermis h	11.22	±1.40	17.22	±2.81	16.83	±2.85
epidermis w	15.07	±3.18	13.35	±2.66	13.77	±2.62
parenchyma h	27.76	±6.34	28.03	±1.52	26.17	±6.55
parenchyma w	30.15	±8.46	51.67	±0.10	32.80	±9.59

Abbreviations: h = height; w = width

10.2. Phytochemical study of the selected *Coffea* species

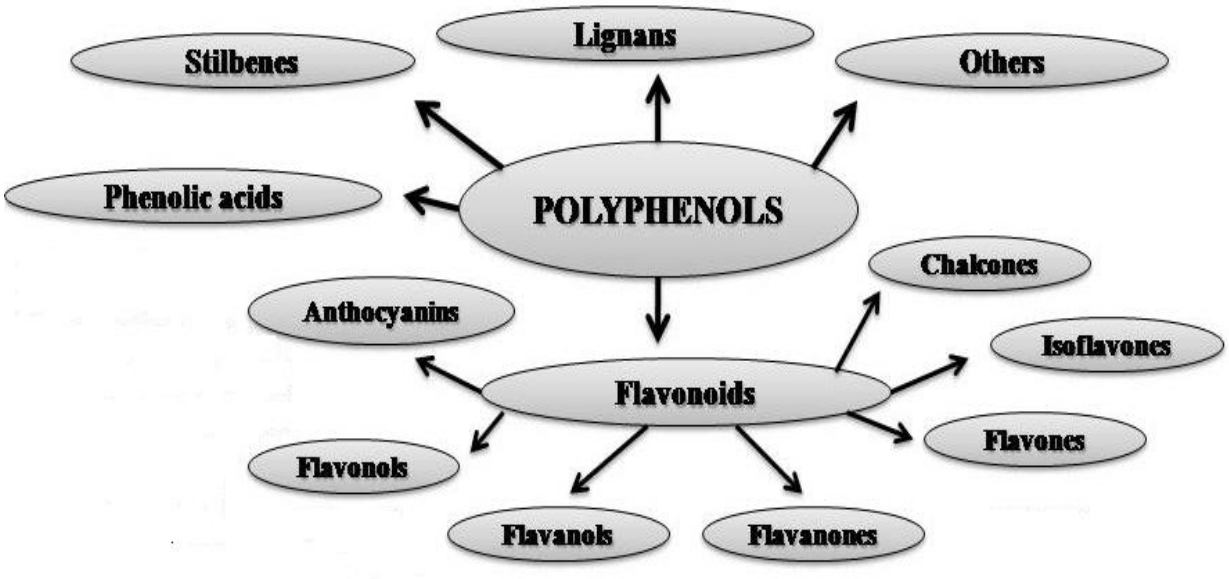


Fig.1. Classification of polyphenols (drawing by Éva Brigitta Patay)

10.2.1. Compounds detected by HPLC-ESI/MS

Table 5. Retention times (t_R) for the investigated polyphenol standards used for HPLC/MS

Peak nr.	Phenolic compound	t_R ± SD (min)	MS method	Equation of calibration curves	Specific ions used for identification Ion [M-H] > Ions from spectrum
1	Caftaric acid *	2.10± 0.06	MRM*	qualitative	311>148.6, 178.6
2	Gentisic acid *	2.15± 0.07	MRM	qualitative	153>108.7
3	Caffeic acid *	5.60± 0.04	MRM	qualitative	179.4>134.7
4	Chlorogenic acid *	5.62± 0.05	MRM	qualitative	353.5>178.7, 190.7
5	*p*-Coumaric acid	8.7± 0.08	MRM	A = - 0.325 + 33.23 x	163> 118.7
6	Ferulic acid	12.2 ± 0.10	MRM	A = - 1.016 + 39.55 x	193.2> 133.7, 148.7, 177.6
6'	*Cis*-ferulic acid	12.5±0.10	MRM	A = - 0.236 + 37.10 x	223.4>148.6, 163.6, 178.7, 207.7
7	Sinapic acid	14.3 ± 0.10	MRM	A = 0.107 + 19.29 x	463.1
8	Hyperoside	18.60 ± 0.12	SIM	A = - 0.273 + 12.97 x	463.1
9	Isoquercitrin	19.60 ± 0.10	SIM	A = 0.226 + 13.47 x	609.1
10	Rutoside	20.20 ± 0.15	SIM	A = -0.544 + 26.45 x	317.1
11	Myricetin	20.70 ± 0.06	SIM	A = 0.241 + 19.19 x	285.1
12	Fisetin	22.60 ± 0.15	SIM	A = 0.047 + 10.69 x	447.1
13	Quercitrin	23.00 ± 0.13	SIM	A = -1.152 + 36.32 x	301.1
14	Quercetol	26.80 ± 0.15	SIM	A= - 0.429 + 31.44 x	331.1
15	Patuletine	28.70 ± 0.12	SIM	A = - 0.760 + 28.97 x	285.1
16	Luteolin	29.10 ± 0.19	SIM	A = - 1.270 + 30.15 x	285.1
17	Kaempferol	31.60 ± 0.17	SIM	A = - 0.908 + 20.40 x	269.2
18	Apigenin	33.10 ± 0.15	SIM	A = - 0.325 + 33.23 x	311>148.6, 178.6

Abbreviation*:* *overlapping in UV detection, only qualitative analysis using MS detection; MRM = multiple reaction monitoring; SIM = single ion monitoring; A = pic area in mAU×s, x = concentration in μg mL^{-1}

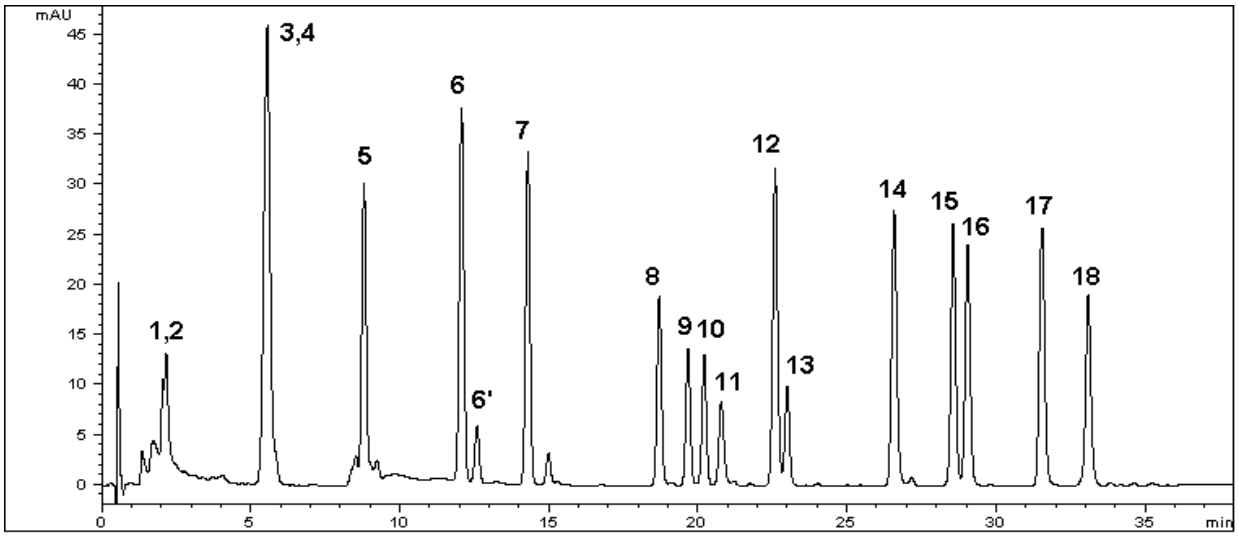


Fig. 2. Chromatogram of the used 18 standards at 330 and 370 nm UV detection

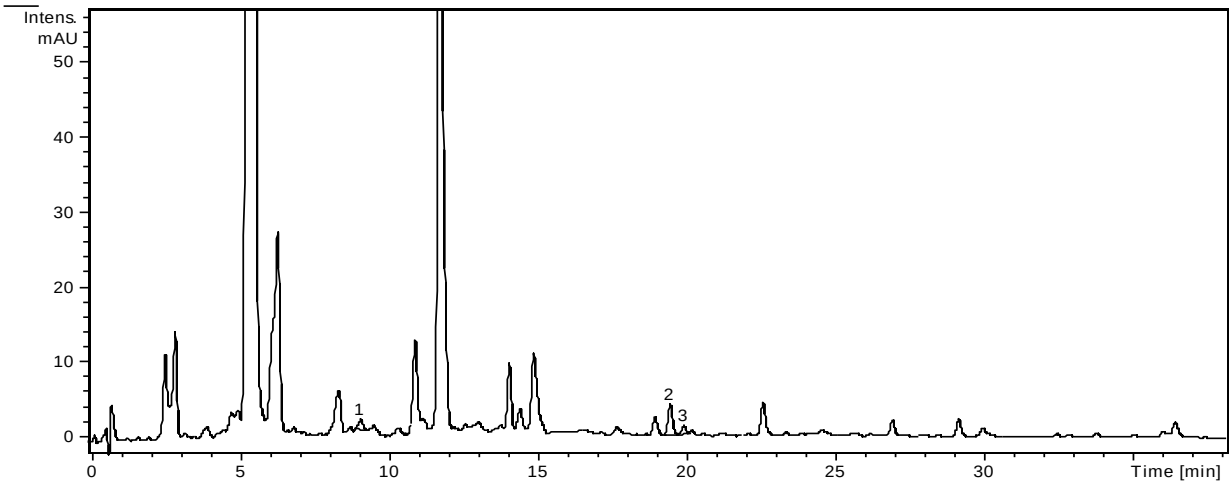


Fig. 3. Chromatogram of the detected polyphenolic compounds in non-hydrolysed extract of the leaf of *C. arabica;* 1: *p*-coumaric acid; 2: isoquercitrin; 3: rutin

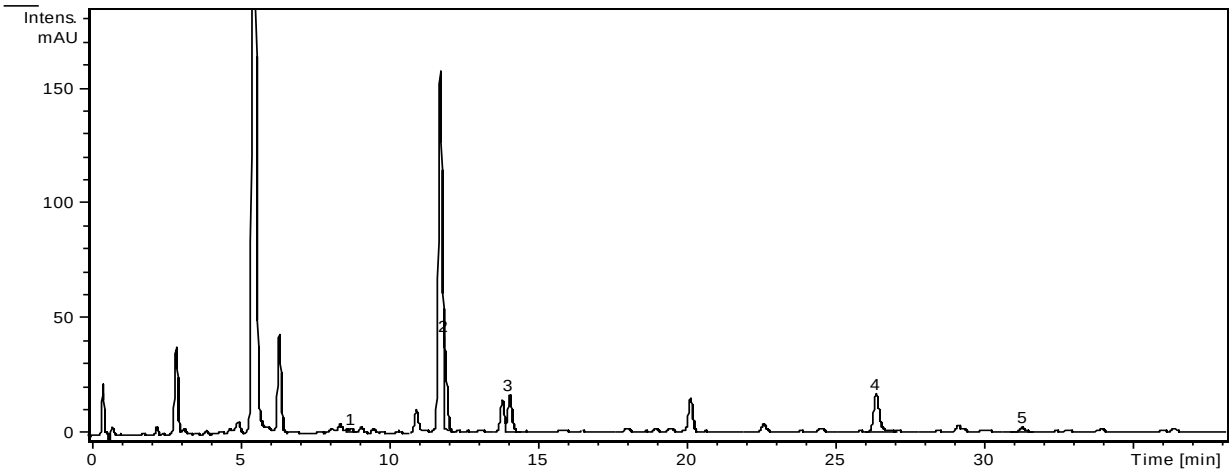


Fig. 4. Chromatogram of the detected polyphenolic compounds in hydrolysed extract of the leaf of *C. arabica*; 1: *p*-coumaric acid; 2: ferulic acid; 3: sinapic acid; 4: quercetin; 5: kaempferol

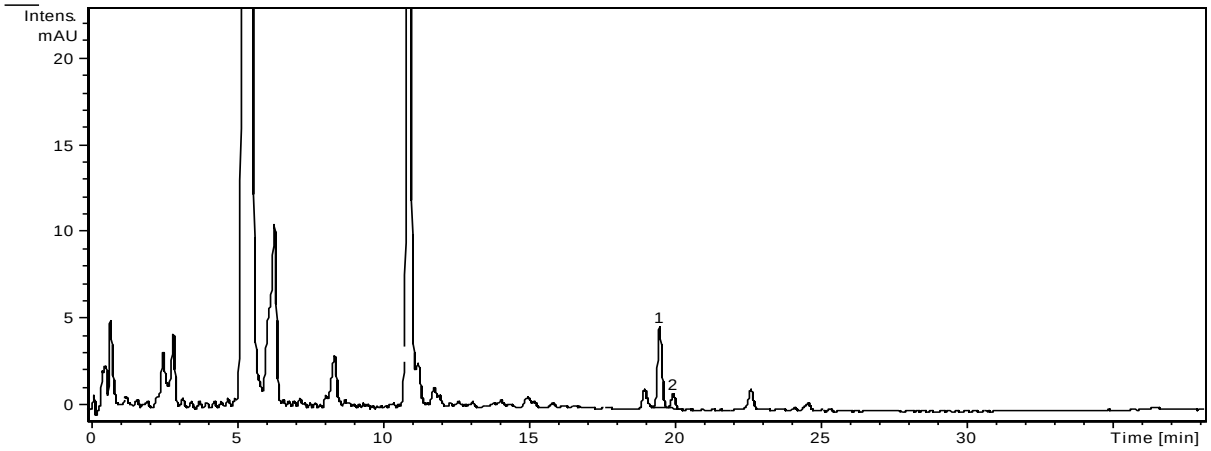


Fig. 5. Chromatogram of the detected polyphenolic compounds in non-hydrolysed extract of the immature pericarp of *C. arabica*; 1: isoquercitrin; 2: rutin

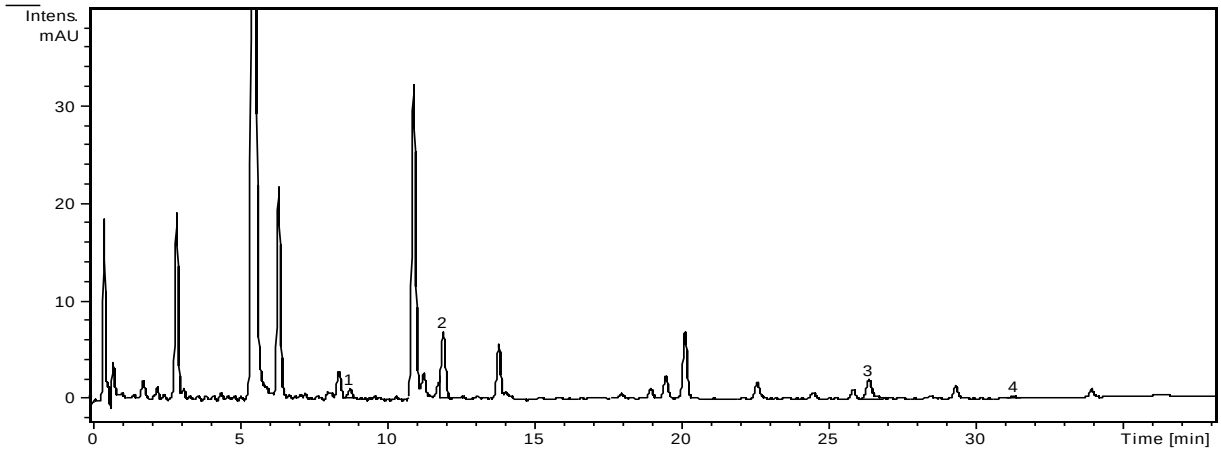


Fig. 6. Chromatogram of the detected polyphenolic compounds in hydrolysed extract of the immature pericarp of *C. arabica*; 1: *p*-coumaric acid; 2: ferulic acid; 3: quercetin; 4: kaempferol

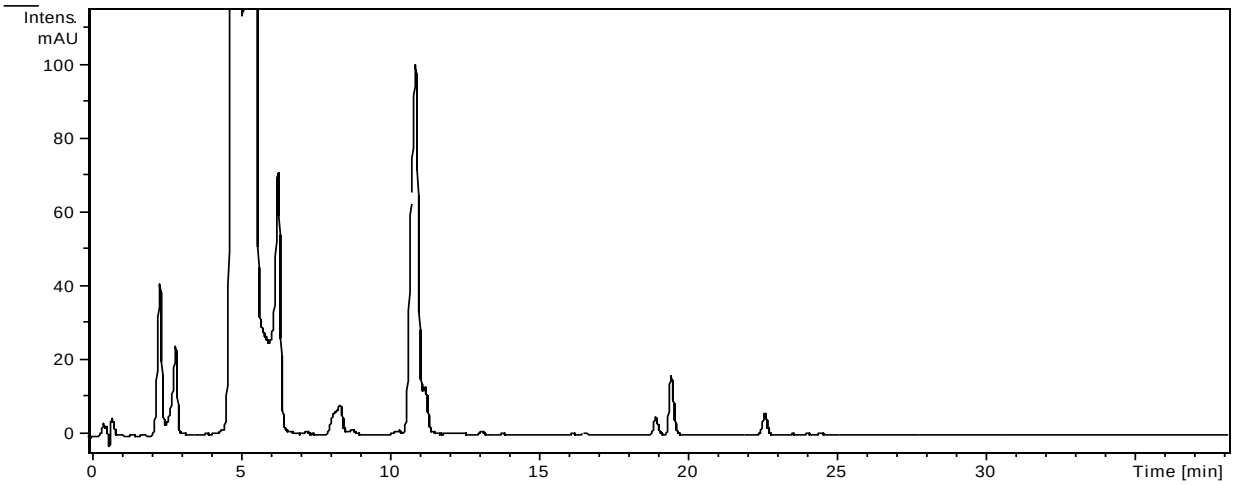


Fig. 7. Chromatogram of the non-hydrolysed extract of the immature seed of *C. arabica;* no polyphenolic compounds were detected

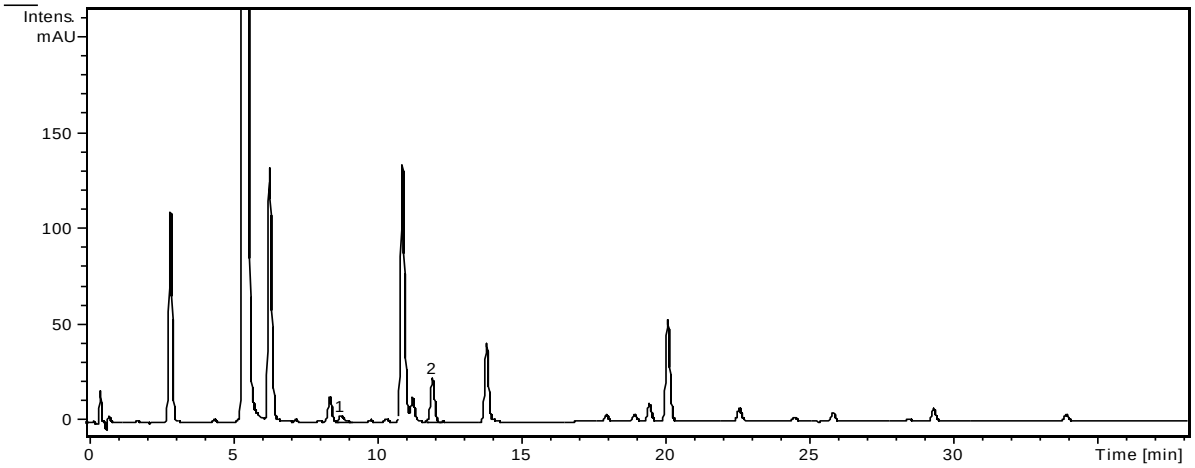


Fig. 8. Chromatogram of the detected polyphenolic compounds in hydrolysed extract of the immature seed of *C. arabica*; 1: *p*-coumaric acid; 2: ferulic acid

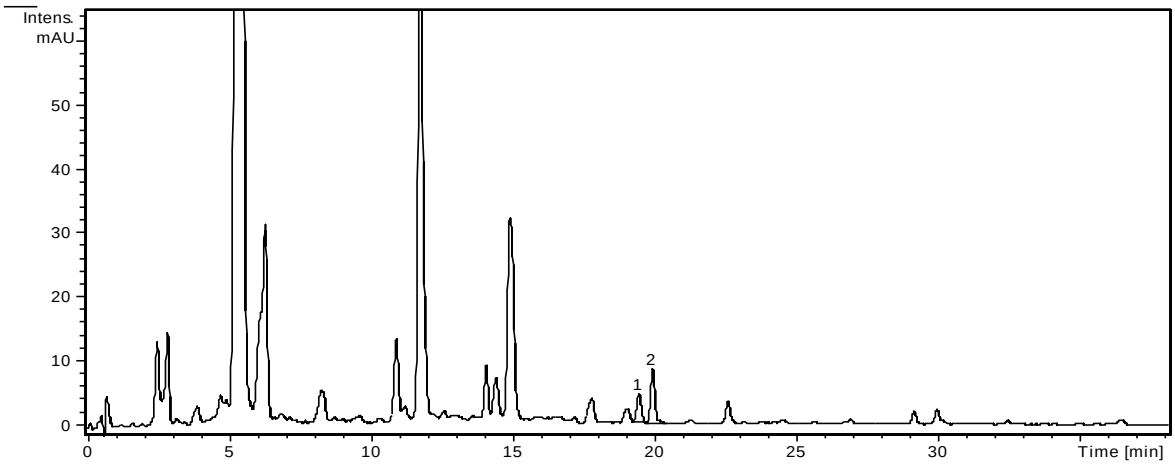


Fig. 9. Chromatogram of the detected polyphenolic compounds in non-hydrolysed extract of the leaf of *C. benghalensis*; 1: isoquercitrin; 2: rutin

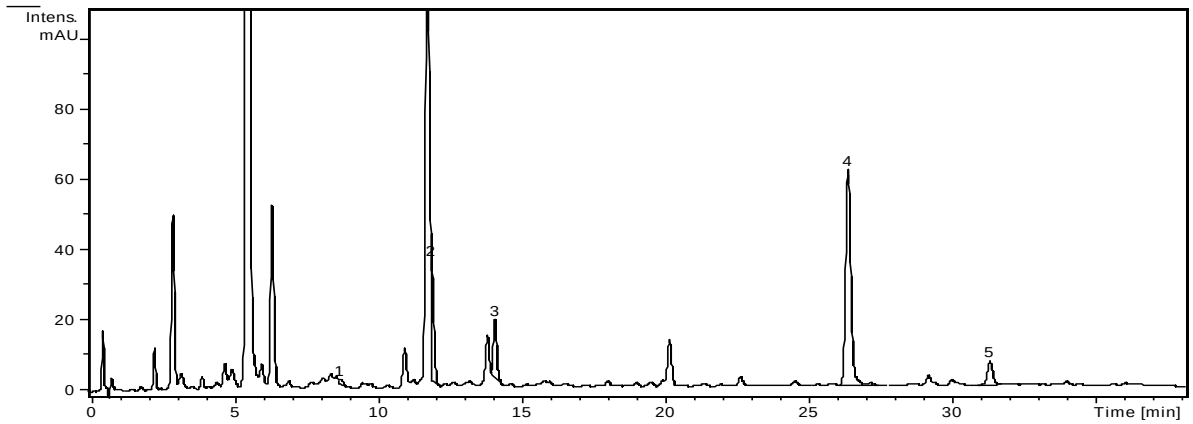


Fig. 10. Chromatogram of the detected polyphenolic compounds in hydrolysed extract of the leaf of *C. benghalensis*; 1: *p*-coumaric acid; 2: ferulic acid; 3: sinapic acid; 4: quercetin; 5: kaempferol

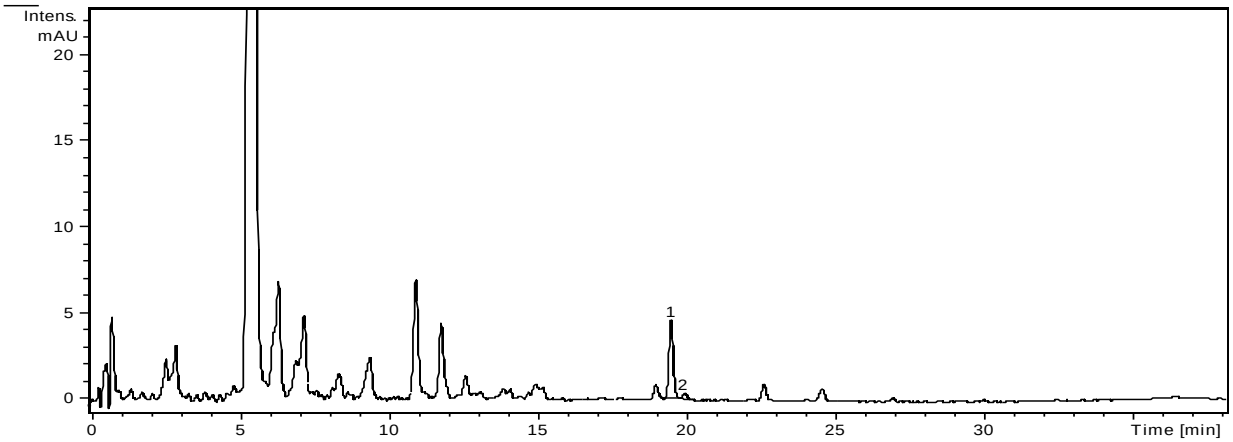


Fig. 11. Chromatogram of the detected polyphenolic compounds in non-hydrolysed extract of the immature pericarp of *C. benghalensis*; 1: isoquercitrin; 2: rutin

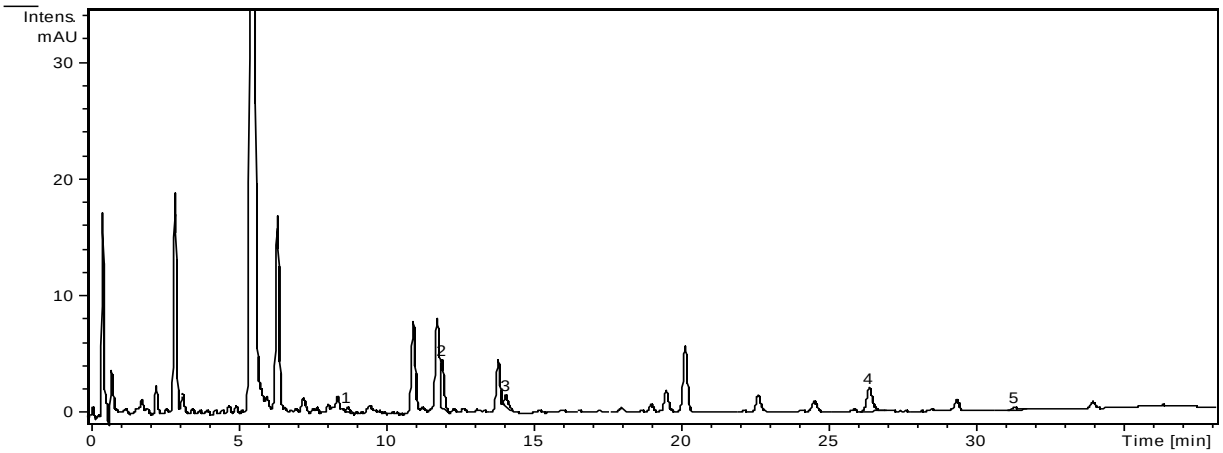


Fig. 12. Chromatogram of the detected polyphenolic compounds in hydrolysed extract of the immature pericarp of *C. benghalensis*; 1: *p*-coumaric acid; 2: ferulic acid; 3: sinapic acid; 4: quercetin; 5: kaempferol

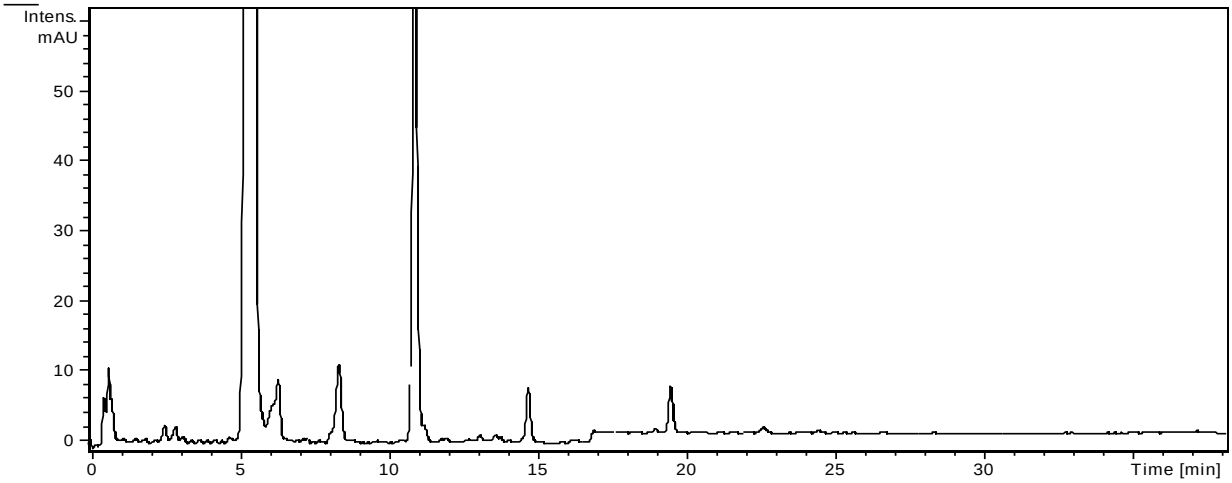


Fig.13. Chromatogram of the non-hydrolysed extract of the immature seed of *C. benghalensis*; no polyphenolic compounds were detected

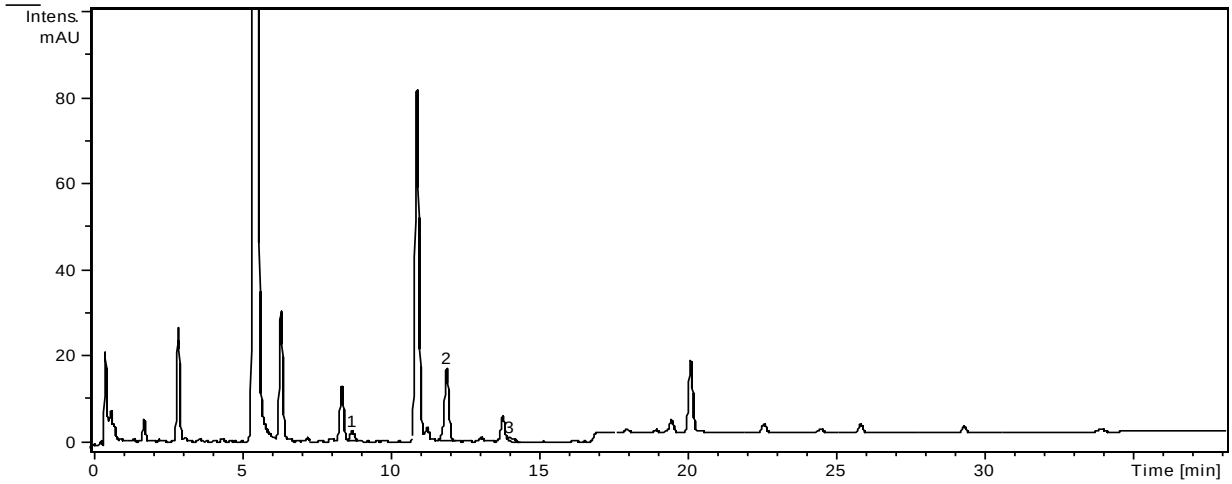


Fig. 14. Chromatogram of the detected polyphenolic compounds in hydrolysed extract of the immature seed of *C. benghalensis*; 1: *p*-coumaric acid; 2: ferulic acid; 3: sinapic acid

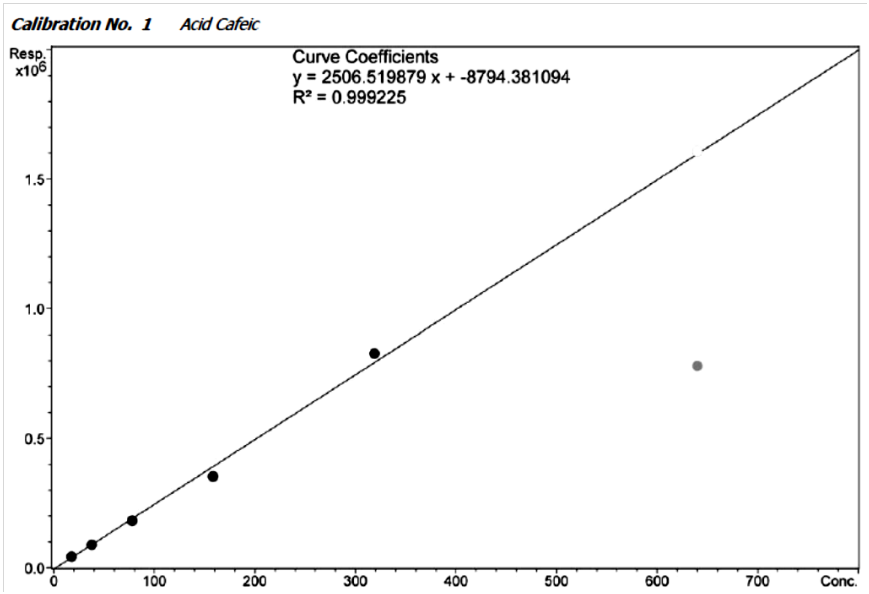


Fig. 15. Calibration curve of caffeic acid for MS/MS detection

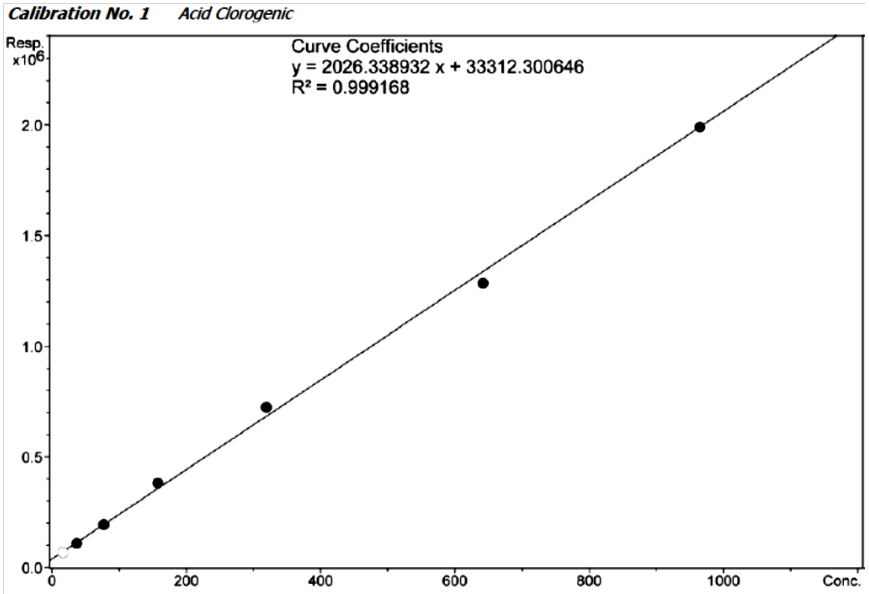


Fig. 16. Calibration curve of chlorogenic acid for MS/MS detection

10.2.2. Compounds detected by HPLC-ESI-MS/MS

Table 6. Studied sample, extract and injected extract quantities

Studied plants and their parts		***Sample quantity (g)***	***Obtained extract (mg/mL)***	***Injected extract (mg/mL)***
C. arabica	leaf	1.090	85.300	0.853
	imature seed	0.800	36.700	0.734
	imature pericarp	0.100	4.330	4.330
	mature seed	1.500	132.700	0.880
	mature pericarp	0.450	47.780	4.780
C. benghalensis	leaf	3.280	268.85	1.780
	imature seed	1.450	36.900	2.460
	imature pericarp	2.400	27.750	1.850
	mature seed	0.500	6.400	6.400
	mature pericarp	0.640	58.400	7.010
C. liberica	leaf	0.980	81.250	8.125
	imature seed	0.980	57.480	5.748
	imature pericarp	0.550	13.700	4.330
	mature seed	0.560	27.775	8.100
	mature pericarp	1.980	173.100	8.655

Table 7. Detected compounds in the leaf of the studied coffees by HPLC-ESI-MS/MS

No.	***Proposed components of the leaf***	**t_R (min)**	**$[M-H]^-$ (*m/z*)**	**MS/MS (*m/z*)**	***C. benghalensis***	***C. arabica***	***C. liberica***
1	isocitric acid	2.3	191	127, 109, 85	+	+	+
3	quinic acid derivate	2.3	533	191	+		
5	3-caffeoylquinic acid	10.2	353	191, 179, 135	+	+	+
9	catechin/epicatechin	12.1	289	245, 203	+		+
10	procyanidin trimer	12.4	863	711, 451, 441	+	+	+
11	procyanidin dimer	12.7	577	451, 425, 407, 287			+
12	5-caffeoylquinic acid	13.1	353	191	+		+
13	4-caffeoylquinic acid	13.6	353	191, 175, 173	+	+	+
14	procyanidin trimer	14.2	863	711, 693, 559, 411, 290	+	+	+
15	catechin/epicatechin	14.7	289	245, 203, 179, 151, 125	+		+
16	5-caffeoylquinic acid	14.9	353	191			+
17	5-coumaroylquinic acid	15.4	337	191			+
18	mangiferin	15.8	421	403, 331, 313, 301, 259	+	+	+
22	3,4-dicaffeoylquinic acid	18.2	515	353, 335, 191, 179, 161	+	+	+
23	3,5-dicaffeoylquinic acid	18.4	515	353, 191	+	+	+
24	4,5-dicaffeoylquinic acid	19.7	515	353, 191, 179	+	+	+
25	ferulic acid	20.3	193	161, 134, 109	+	+	+

Table 8. Detected compounds in the immature pericarp of the studied coffee species by HPLC-ESI-MS/MS

No.	***Proposed components of the immature pericarp***	**t_R (min)**	**$[M-H]^-$ (*m/z*)**	**MS/MS (*m/z*)**	***C. benghalensis***	***C. arabica***	***C. liberica***
1	isocitric acid	2.3	191	109, 93, 85, 81, 71	+	+	+
5	3-caffeoylquinic acid	10.2	353	191, 179, 135		+	+
6	procyanidin dimer	10.2	577	451, 425, 407, 289, 245		+	
7	4-caffeoylquinic acid	11.6	353	191, 179, 173		+	
9	catechin/epicatechin	12.1	289	245, 203, 179, 175, 151		+	+
11	procyanidin dimer	12.7	577	425, 407, 289, 245, 161			+
12	5-caffeoylquinic acid	13.1	353	191	+	+	+
13	4-caffeoylquinic acid	13.6	353	191, 179, 175, 173, 135	+	+	+
15	catechin/epicatechin	14.7	289	245, 203, 179, 175, 151	+	+	+
16	5-caffeoylquinic acid	14.9	353	191		+	+
17	5-coumaroylquinic acid	15.4	337	191, 173		+	
18	mangiferin	15.8	421	403, 331, 313, 301, 259		+	+
19	4-feruloylquinic acid	15.9	367	191, 173, 134		+	+
21	5-feruloylquinic acid	17.4	367	191		+	
22	3,4-dicaffeoylquinic acid	18.2	515	353, 191, 179, 173, 161		+	+
23	3,5-dicaffeoylquinic acid	18.4	515	353, 191, 179, 173		+	+
24	4,5-dicaffeoylquinic acid	19.7	515	353, 191, 179, 173, 135		+	+
25	ferulic acid	20.3	193	161, 133		+	

Table 9. Detected compounds in the immature seed of the studied coffee species by HPLC-ESI-MS/MS

No.	***Proposed components of the immature seed***	**t_R (min)**	**$[M-H]^-$ (*m/z*)**	**MS/MS (*m/z*)**	***C. benghalensis***	***C. arabica***	***C. liberica***
1	isocitric acid	2.3	191	109, 93, 85, 81, 71	+	+	+
2	caffeoyl hexoside	2.3	341	179, 119, 113	+	+	
5	3-caffeoylquinic acid	10.2	353	191, 179, 135	+	+	+
8	5-caffeoylquinic acid derivative	12.1	385	353, 191		+	+
11	procyanidin dimer	12.7	577	191, 179, 173			+
12	5-caffeoylquinic acid	13.1	353	191, 173, 135	+	+	+
13	4-caffeoylquinic acid	13.6	353	191	+	+	+
15	catechin/epicatechin	14.7	289	191, 175, 173, 135	+		+
16	5-caffeoylquinic acid	14.9	353	245, 203, 179, 125		+	+
17	5-coumaroylquinic acid	15.4	337	191		+	+
19	4-feruloylquinic acid	15.9	367	191, 173	+	+	+
20	5-caffeoylquinic acid methyl ether	16.8	367	179, 161, 135			+
21	5-feruloylquinic acid	17.4	367	191		+	+
22	3,4-dicaffeoylquinic acid	18.2	515	353, 335, 179, 173	+	+	+
23	3,5-dicaffeoylquinic acid	18.4	515	353, 191, 179, 173	+	+	
24	4,5-dicaffeoylquinic acid	19.7	515	353, 191, 179, 173	+	+	+
25	ferulic acid	20.3	193	161, 133, 131	+	+	+

Table 10. Detected compounds in the mature pericarp of the studied coffee species by HPLC-ESI-MS/MS

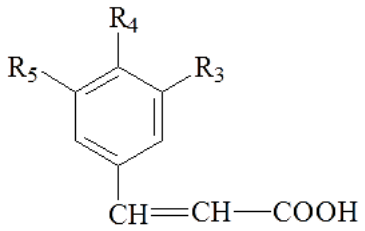
No.	***Proposed components of the mature pericarp***	**t_R (min)**	**$[M-H]^-$ (*m/z*)**	**MS/MS (*m/z*)**	***C. benghalensis***	***C. arabica***	***C. liberica***
1	isocitric acid	2.3	191	-	+	+	+
2	caffeoyl hexoside	2.3	341	179, 119, 89		+	
3	quinic acidderivate	2.3	533	191, 127		+	
5	3-caffeoylquinic acid	10.2	353	191, 179, 135	+	+	
8	5-caffeoylquinic acid derivative	12.1	385	353, 191	+		
9	catechin/epicatechin	12.1	289	245, 203, 221, 109	+		
11	procyanidin dimer	12.7	577	425, 407, 289, 125	+		
12	5-caffeoylquinic acid	13.1	353	191	+	+	+
13	4-caffeoylquinic acid	13.6	353	191, 175, 173, 135	+	+	+
15	catechin/epicatechin	14.7	289	245, 203, 179, 151, 125	+	+	+
16	5-caffeoylquinic acid	14.9	353	191	+	+	
17	5-coumaroylquinic acid	15.4	337	191	+	+	
18	mangiferin	15.8	421	331, 301, 259	+	+	
19	4-feruloylquinic acid	15.9	367	191, 173	+	+	
22	3,4-dicaffeoylquinic acid	18.2	515	353, 335, 179, 173	+	+	
23	3,5-dicaffeoylquinic acid	18.4	515	353, 191, 179	+	+	
24	4,5-dicaffeoylquinic acid	19.7	515	353, 179, 173	+	+	+
25	ferulic acid	20.3	193	161, 133,	+	+	

Table 11. Detected compounds in the mature seed of the studied coffee species by HPLC-ESI-MS/MS

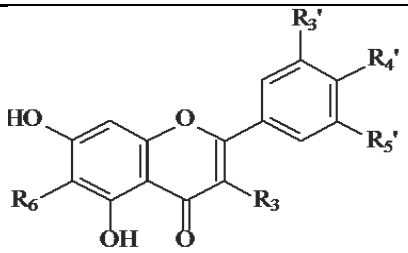
No.	***Proposed components of the mature seed***	**t_R (min)**	**$[M-H]^-$ (*m/z*)**	**MS/MS (*m/z*)**	***C. benghalensis***	***C. arabica***	***C. liberica***
1	isocitric acid	2.3	191	127, 109, 93, 85	+	+	+
2	caffeoyl hexoside	2.3	341	179, 119		+	
4	citric acid	3.0	191	111, 87, 67			+
5	3-caffeoylquinic acid	10.2	353	191, 179, 135		+	+
7	4-caffeoylquinic acid	11.6	353	191, 179, 173			+
8	5-caffeoylquinic acidderivative	12.1	385	353, 191			+
12	5-caffeoylquinic acid	13.1	353	191	+	+	+
13	4-caffeoylquinic acid	13.6	353	191, 179, 173	+	+	+
15	catechin/epicatechin	14.7	289	245, 203, 125	+		
16	5-caffeoylquinic acid	14.9	353	191	+	+	+
17	5-coumaroylquinic acid	15.4	337	191, 173		+	+
18	mangiferin	15.8	421	331, 301, 259	+		
19	4-feruloylquinic acid	15.9	367	191, 173	+	+	+
20	5-caffeoylquinic acid methyl ether	16.8	367	179, 135			+
21	5-feruloylquinic acid	17.4	367	191	+		
22	3,4-dicaffeoylquinic acid	18.2	515	353, 335, 191, 179, 173	+	+	+
23	3,5-dicaffeoylquinic acid	18.4	515	353, 191			+
24	4,5-dicaffeoylquinic acid	19.7	515	353, 191, 179, 173, 135	+	+	+
25	ferulic acid	20.3	193	161, 133	+		+

10.2.3. Chemical structure of the most important detected compounds

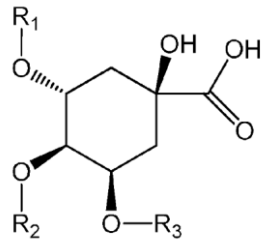
Table 12. Chemical structure of the detected compounds



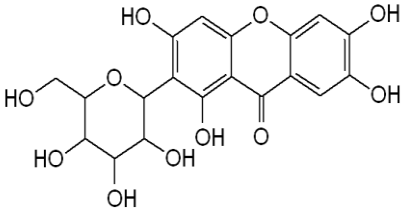
	R_3	R_4	R_5
p-Coumaric acid	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	H	OH	OCH_3
Sinapic acid	OCH_3	OH	OCH_3
Chlorogenic acid	OH	OH	H



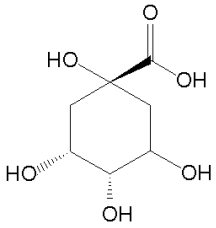
	R_3	R_6	$R_{3'}$	$R_{4'}$	$R_{5'}$
Isoquercitrin	O-β-D-glucoside	H	OH	OH	H
Quercetin	O-β-D-rhamnoside	H	OH	OH	H
Rutin	O-β-D-rutinoside	H	OH	OH	H
Kaempferol	OH	H	OH	H	H



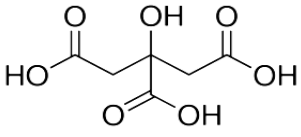
	R_1	R_2	R_3
4-feruloylquinic acid	H	feruloyl	H
5-feruloylquinic acid	H	H	feruloyl
5-coumaroylquinic acid	H	H	cumaroyl
3-caffeoylquinic acid	caffeoyl	H	H
4-caffeoylquinic acid	H	caffeoyl	H
5-caffeoylquinic acid	H	H	caffeoyl
3,4-dicaffeoylquinic acid	caffeoyl	caffeoyl	H
3,5-dicaffeoylquinic acid	caffeoyl	H	caffeoyl
4,5-dicaffeoylquinic acid	H	caffeoyl	caffeoyl



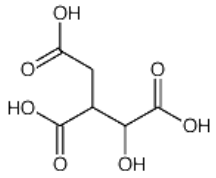
Mangiferin



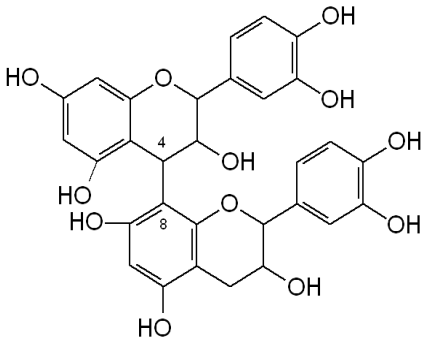
Quinic acid



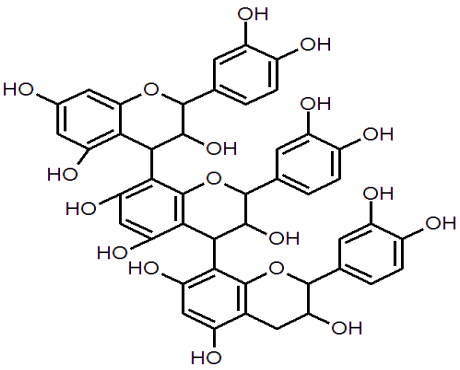
Citric acid



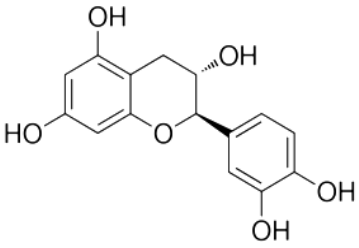
Isocitric acid



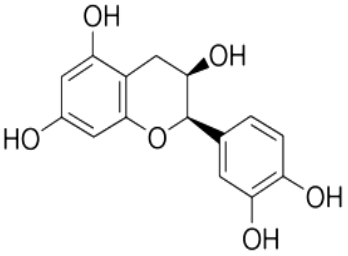
Procyanidin dimer



Procyanidin trimer



Catechin



Epicatechin

11. Publications, posters and oral presentations (imported from MTMT)

2017

1. Eva Brigitta Patay, Fritea Luminita, Andreea Antonescu, Angela Antonescu, Luciana Dobjanschi
   Jolanta Latosińska, Magdalena Latosińska (szerk.)
   Caffeine Research: *Coffea arabica* - a plant with rich content in caffeine
   Rijeka: InTech Open Access Publisher, 2017.
   (ISBN:978-953-51-5216-3)
   Könyv /Szakkönyv /Tudományos
   In Press

2. Papp Nora, Balázs Viktoria Lilla, Bartha Samuel Gergely, Bencsik Timea, Dénes Tunde , Filep Rita, Gyergyák Kinga, Patay Éva Brigitta , Joós-Békésiné Kallenberger Helena, Tóth Monika, Farkas Ágnes
   Gyógynövények hisztológiai értékelése- oktatás és kutatás a pécsi Farmakognóziai Intézetben
   Konferencia helye, ideje: Budapest, Magyarország, 2017.09.07.
   Budapest: 2017.
   (ISBN:978-963-12-9834-5)
   Egyéb konferenciakötet / /Tudományos

2016

3. Éva Brigitta Patay, Nikolett Sali, Tamás Kőszegi, Rita Csepregi, Viktória Lilla Balázs, Tibor Sebastian Németh, Tibor Németh, Nóra Papp
   Antioxidant potential, tannin and polyphenol contents of seed and pericarp of three *Coffea* species
   ASIAN PACIFIC JOURNAL OF TROPICAL MEDICINE 9:(4) pp. 366-371. (2016)
   Link(ek): DOI, PubMed, WoS, Scopus
   Folyóiratcikk /Szakcikk /Tudományos
   Független idéző: 2 Függő idéző: 1 Összesen: 3
   1 * *Patay Eva Brigitta, Bencsik Timea, Papp Nora*
   Phytochemical overview and medicinal importance of *Coffea* species from the past until now
   ASIAN PACIFIC JOURNAL OF TROPICAL MEDICINE (ISSN: 1995-7645) 9: (12) *pp. 1101-1110. (2016)*
   Link(ek): DOI, PubMed, WoS, Scopus
   Folyóiratcikk /Összefoglaló cikk /Tudományos
   2 *Mahmoud AH, Soliman MS, Metwally NS, Farrag AH, Elsharabasy FS, Arafa S, Ibrahim AMM*
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