DNA fingerprinting and profile of phenolics in root and root calli of *Arctium lappa* L. grown in Egypt

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Aim

The aim of this study was the establishment of an efficient and promising protocol for callus production from *Arctium lappa* L. roots (family *Asteraceae*) and comparison of the metabolic profile of their phenolic and flavonoid content. DNA fingerprinting of *A. lappa* L. was carried out using the molecular generic marker technique (random amplification of polymorphic DNA-PCR), which was newly introduced in Egypt, for identification and authentication of the plant.

Methods

The effect of different concentrations of benzyladenine and naphthalene acetic acid added to MS media on initiation of root callus production and mass of callus produced was investigated. The presence or absence of various secondary metabolites of the root and calli was also determined using colorimetric methods and high performance liquid chromatography.

Results and conclusion

The growth parameters of the callus were determined. Each callus differs from the root in the profile of phenolic and flavonoid content. The calli have a higher phenolic content than the root and differ in the flavonoid profile.

Keywords:

Arctium lappa L, DNA fingerprinting, flavonoids, phenolics, root callus

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Introduction

Arctium lappa L. or burdock (*Asteraceae*) is native to Europe and north Asia. Traditionally, it has been used as a safe and edible food product [1,2] and for the treatment different ailments [3–5]. Phytochemical investigation of different organs of the plant revealed the presence of fixed oil, phenolic acids, flavonoids, lignans [2,6], resin, mucilage, essential oil [7], polyacetylenes [7], and caffeoylquinic acid derivatives [8]. In a previous study [9], bioactive lignans and phenolics and the biological activities of extracts from different organs of *A. lappa* L. cultivated in Egypt were studied. PCR sequencing was carried out for six *A. lappa* L. breeds from southern Taiwan using two primers, ITS1-5.8S and rRNA-ITS2, which revealed that they all had an amplified fragment that was 358 bp in length [10]. Automatic sequence analysis showed that the DNA sequences for different breeds of *Arctium* can differ [10]. Hypocotyls and cotyls of the plant were induced to produce callus for high frequency plant regeneration [11]. In the current literature, few studies on tissue culture and DNA fingerprinting of the plant were found, but no reports dealing with the phenolic profile of the callus were found. Accordingly, the aim of the present work was to carry out PCR sequencing for the identification and authentication of *A. lappa* L., a plant grown in Egypt, and to study the root callus metabolites, as the accumulation of secondary products in plant cell cultures depends on the composition of the culture medium.

Subjects and methods

Plant material

Authentic seeds of *A. lappa* L. were kindly provided to Prof. Dr E.A. Aboutabl by the Botanic Garden, Bonn, Germany and were cultivated in the Experimental Station of Medicinal and Aromatic Plants, Faculty of Pharmacy, Cairo University. For tissue culture, seeds were collected from the cultivated plant during the fruiting stage.

Plant material for DNA fingerprinting

Freeze-dried leaves of *A. lappa* (10 g) were powdered in liquid nitrogen, and genomic DNA was extracted by a modification of the cetyltrimethylammonium bromide method [12].

Reference standards

Rutin, daidzein, genistein, isorhamnetin, luteolin, biochanin A, hyperoside, gallic acid, chlorogenic acid, caffeic acid, ferulic acid, and coumarin were obtained from the Department of National Organization of Drug Control and Research Standards.

Primers were obtained from Operon Technologies Inc. (Almeda, California, USA).

Methods

DNA amplification was carried out using the random amplification of polymorphic DNA (RAPD) technique

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Table 1 Sequence of 15 primers assayed using the RAPD-PCR technique

Primer	Sequences (5′–3′)
A-01	5′-CAGGCCCTTC-3′
A-11	5′-CAATCGCCGT-3′
B-06	5′-TGCTCTGCCC-3′
B-08	5′-GTCCACACGG-3′
B-15	5′-GGAGGGTGTT-3′
B-18	5′-CCACAGCAGT-3′
P-01	5′-GTAGCACTCC-3′
O-02	5′-ACGTAGCGTC-3′
O-09	5′-TCCCACGCAA-3′
E-08	5′-TCACCACGGT-3′
E-05	5′-TCACCACGGT-3′
E-11	5′-GAGTCTCAGG-3′
G-06	5′-GTGCCTAACC-3′
Z-13	5′-GACTAAGCCC-3′
G-17	5'- ACGACCGACA-3'

RAPD, random amplification of polymorphic DNA.

with 15 primers (the sequences are shown in Table 1). The GeneAmp PCR system 9700 (Perkin Elmer, Cambridge, UK) and a gel documentation system (Bio-Rad Gel Doc-2000, Bio-Rad Laboratories, GmbH, Munich, Germany) were used for photographing of PCR products.

PCR reactions[13,14] were carried out in a total volume of 25 μl with 10 ng/μl of genomic DNA as a template, 3 μl of random primer, 2.5 μl of 2 mmol/l dNTP mix (Abgene, Surrey, UK), 2.5 μl of 10 × PCR buffer, 2 μl of 25 mmol/l $MgCl_2$, and 0.3–5 U/μl of Taq DNA polymerase. An aliquot of 22 μl of master mix solution was dispensed in each PCR tube (0.2 ml Eppendorf tube) containing 3 μl of the appropriate template DNA. The reaction involved initial denaturation by heating for 4 min at 94°C. Complete denaturation of DNA indicated efficient utilization of the template in the first amplification cycle and a good yield of the PCR product. The reaction mixture was then subjected to 40 cycles of the following program: a denaturation step at 94°C for 45 s, an annealing step at 36°C for 1 min, and an elongation or extension step at 72°C for 2 min. After the last cycle, the mixture was subjected to a final extension step for 7 min at 72°C, followed by soaking at 4°C until removal of the reaction mixture from the PCR machine. The amplification products were resolved by electrophoresis on a 1.4% agarose gel containing ethidium bromide (0.5 μg/ml) in 1 × tris-borate-EDTA buffer. A total of 15 μl of each PCR product was mixed with 3 μl of loading buffer (tracking dye) and loaded into the wells of the gel. The gel was run at 85 V for about 3 h or until the tracking dye reached the gel. An ultraviolet (UV) Polaroid camera was used for visualization of RAPD. Polaroid camera was used for 6 visualization of RAPD; markers being scored as DNA fragments present in some lanes and absent in others.

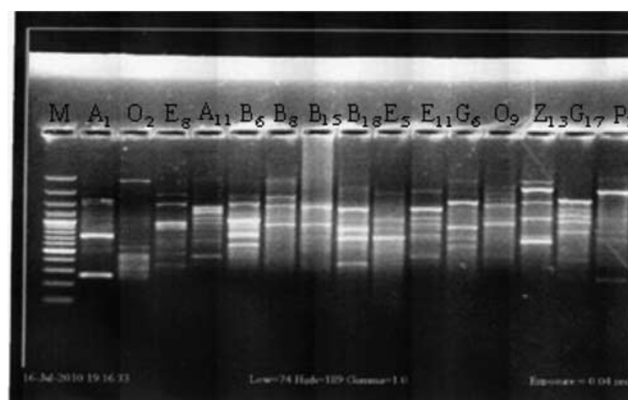
Tissue culture

The seeds were washed thoroughly with running tap water for about 15 min and surface-sterilized by immersion in 10% commercial Savlon solution (10th of Ramadan, Sharkiah, Egypt) (an antiseptic solution containing 0.3% w/v chlorhexidine gluconate and 3% w/v cetrimide) for about 5 min with shaking. The seeds were then washed three times with sterile distilled water and immersed in 30% commercial Clorox solution (10th of Ramadan) (a disinfectant containing 1.5% sodium hypochlorite) with 1–2 drops of wetting agent (Tween 80) while shaking on a shaker for 10 min. Thereafter, the seeds were washed three times with sterile distilled water. They were then cultured in a jar containing sterile solid MS control media without a plant growth regulator and incubated at 22–28°C with a photoperiod of 16 h/day (200–2500 lx). After 6–8 weeks, the plantlets grown were used to obtain the explants used for callus cultures. The 6–8-week-old seedlings grown *in vitro* on sterile MS medium (Fig. 1) were used as a sources of explants [15]. Dissection of uniformly-sized explants (about 0.5 cm in length) from different organs – that is, shoot tips, leaves, roots, and stems – was performed under aseptic conditions using a sterile scalpel and forceps [16]. The different explants were cultured in jars containing sterile medium supplemented with different concentrations of various plant growth regulators such as benzyladenine (BA), kinetin, naphthalene acetic acid (NAA), indolebutyric acid, 2,4-dichlorophenoxy acetic acid, and indole acetic acid. For each condition, 30 jars were prepared, each jar containing five explants. The cultures were incubated at 21°C (± 2°C) with a photoperiod of 16 h/day (1500–2000 lx) for a period of 6 weeks.

Determination of total phenolics

Air-dried plant root calli 1 and 2 (1 g each) were defatted with petroleum ether and extracted with 70% methanol by sonication at room temperature. Stock solution (concentration: 1 mg/ml) was prepared from the concentrated residue by dissolving in distilled water. The phenolic compound in the root was found to be gallic acid on Folin–Ciocalteau colorimetry [17] using a Shimadzu 1601 spectrophotometer at 730 nm, and the total phenolic content of the root was compared with that

Figure 1



The random amplification of polymorphic DNA electrophoretic profile of *Arctium lappa* L., cultivated in Egypt, generated by 15 primers (M: 100 bp plus fermentas).

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of the calli. Determination of total the phenolic content of the cultivated roots, leaves, and seeds was carried out in our previous work [9].

Colorimetric determination of total flavonoid content

Powdered, air-dried (2 g) plant root calli 1 and 2 were defatted with petroleum ether, extracted with 70% methanol till exhaustion, and evaporated to dryness. The combined methanolic extract was adjusted to 50 ml. A 5 ml aliquot of each extract was treated with a 5 ml aliquot of 0.1 mol/l $AlCl_3$ reagent [18]. The absorbance of the color developed was measured at λ_{max} 422 nm against a blank, and the corresponding amount of rutin was recorded.

HPLC determination of isoflavones

Dried root (1 g) and root calli (1 and 2, 0.25 g) were separately defatted, filtered, and extracted with 50% ethanol. The ethanol was evaporated under vacuum at 35°C, and the phenolics in the remaining aqueous solution were extracted with ethyl acetate (1 : 1). The phenolic fractions were stored in the dark at 4°C until analysis by high performance liquid chromatography (HPLC). An Aglient 1100 system (Agilent Technologies Deutschland GmbH, Germany) equipped with a column compartment, quaternary pump, degasser, auto sampler, and UV detector was used for HPLC analysis. Elution was performed at a flow rate of 1 ml/min with a mobile phase of water/acetic acid (98 : 2 v/v, solvent A) and methanol/ acetonitrile (50 : 50 v/v, solvent B), starting with 5% B and increasing the level of B to 30% at 25 min, 40% at 35 min, 52% at 40 min, 70% at 50 min, and 100% at 55 min; the UV detector was set at 254 nm [19]. Retention times were compared with those of certain standard isoflavones. Before injection into the HPLC system, each sample was filtered through a 0.4 μm membrane filter into the sample vial for injection.

HPLC determination of phenolics

Extraction and HPLC analysis of phenolics were carried out under the same conditions as those for isoflavones, but measurements were made with a detector set at 330 nm. Retention times were compared with those of available phenolic standards.

Results

Total genomic DNA profiling of *A. lappa* L., grown in Egypt, was performed using 15 random primers. The number of banding patterns generated by each primer was recorded to obtain the DNA profile of *A. lappa* under investigation, in order to compare it with previously reported phenotypic characters as well as for chemical investigations. Molecular size, in base pairs, of amplified DNA fragments produced by 15 decamer primers in *A. lappa* L. is listed in Table 1, and their reproducible RAPD profiles generated are shown in Fig. 1. The total number of bands generated by the 15 primers was 93, the smallest size of amplified product being 245 bp, whereas the largest size of the amplified product being 3030 bp. Primer P1 produced nine bands, with 245 bp being the smallest size and 3030 bp being the largest size; primer A1 was the least reproducible and generated three bands with molecular sizes 1739, 724, and 276 bp.

Callus production

Figure 2 shows 6–8-week-old seedlings of *A. lappa* grown *in vitro* on sterile MS media; these were used as a source of explants for callus production. Trials using different explants (shoot tips, leaves, stems, and roots) and different growth regulators were carried out for initiation of callus. Calli were obtained successfully on MS media supplemented with plant growth regulators for roots: MS + 0.5 mg/l BA + 1 mg/l NAA (callus 1) and MS + 0.5 mg/l BA + 0.1 mg/l NAA (callus 2). The different callus

Figure 2

Six- to eight-week-old seedling of *Arctium lappa* L. grown *in vitro* in MS medium.

Table 2 The effect of plant growth regulators on callus growth parameters of *Arctium lappa* L. root and total phenolic and flavonoid content of *Arctium lappa* L. root and two calli

		Root calli (greenish brown compact undifferentiated callus)	
Characteristics	Root	Callus 1	Callus 2
Callus fresh weight (g)	–	5.01 + 0.3	4.22 + 0.2
Callus dry weight (g)	–	0.48 + 0.05	0.36 + 0.03
Total phenolic content (%; calculated as gallic acid content in dried material)	5.33	6.53	7.98
Total flavonoid content (%; calculated as rutin content in dried material)	0.05	0.003	0.002

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Figure 3

(a) Callus 1 (×0.76; MS + 0.5 mg/l BA + 1 mg/l NAA). (b) Callus 2 (×1; MS + 0.5 mg/l BA + 0.1 mg/l NAA).

growth parameters are listed in Table 2, and callus types are presented in Fig. 3a and b.

Determination of total phenolic content of the root calli compared with that of the root

Colorimetric determination showed that there was a variation in the phenolic content of the root compared with that of the calli (Table 2). Callus 2 showed a higher phenolic content than callus 1 and the root because of the effect of plant growth regulators (BA and NAA) on the biosynthesis of polyphenols [15].

Determination of the flavonoid compounds of the root calli compared with those of the *A. lappa* L. root

The flavonoid content in each of the two calli was less than that in the root (Table 2).

HPLC determination of isoflavones in the root callus compared with those in the main plant parts

The concentration of isoflavones (in mg/g; Table 3) indicates that the root contains only genistein and differs in metabolic profile compared with root callus 1 (MS + 0.5 mg/l BA + 1 mg/l NAA), which contains isorhamnetin and biochanin A, and root callus 2 (MS + 0.5 mg/l BA + 0.1 mg/l NAA), which contains daidzein and genistein. The flavonoid content in callus culture differs qualitatively and quantitatively from that in the parent plant [20].

Table 3 Phenolics identified by high performance liquid chromatography in *Arctium lappa* L. root and calli

		Concentration (mg/g)		
Compounds	R_t (min)	Root	Callus 1	Callus 2
Gallic acid	2.51	0.49	0.36	0.78
Daidzein	3.03	–	–	0.054
Genistein	3.57	0.005	–	0.014
Isorhamnetin	4.29	–	0.080	–
Chlorogenic acid	5.54	0.62	0.06	0.58
Caffeic acid	6.15	–	0.06	0.70
Biochanin A	7.17	–	0.018	–
Hyperoside	7.95	0.31	–	–
Rutin	8.11	0.22	–	–
Ferulic acid	9.17	0.01	0.06	–
Coumarin	9.70	0.02	0.22	0.66
Luteolin	11.78	0.01	–	0.62

HPLC determination of the phenolic content of the root callus compared with that of the root

The root differs in its phenolic metabolic profile compared with the two calli. The phenolic compounds present in the root were identified as gallic acid, ferulic acid, chlorogenic acid, hyperoside, rutin, coumarin, and luteolin. Callus 1 was found to contain gallic acid, chlorogenic acid, caffeic acid, ferulic acid, and coumarin, whereas callus 2 was found to contain gallic acid, chlorogenic acid, caffeic acid, coumarin, and luteolin. The corresponding concentrations are listed in Table 3 (in mg/g; Table 4).

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Table 4 Molecular size, in base pairs, of amplified DNA fragments produced by 15 decamer primers in *Arctium lappa* L.

Molecular size of DNA marker (bp)	A_1	A_{11}	O_2	O_9	E_5	E_8	E_{11}	B_6	B_8	B_{15}	B_{18}	G_6	G_{17}	Z_{13}	p_1
245													+		+
268			+												
276	+														
310			+												
359						+									
370											+				
440			+				+								
453		+													
467						+									
525										+		+			
556			+												
573						+									
590								+							
644		+				+								+	
683							+				+				+
703					+					+		+		+	
724	+														
745						+	+	+							
813													+		
838															+
862											+				
888					+	+	+								
914												+			
941								+							
969									+	+					
998		+		+		+	+								
1028													+		
1058		+						+			+				
1090					+										+
1122		+					+							+	
1155				+											
1189									+				+		
1225								+							
1298		+													+
1337				+											
1377													+		
1417		+					+			+	+				
1503															
1547				+					+						
1593					+	+		+							
1640												+		+	+
1989													+		
1739	+														
1791				+											
1844															+
1899								+		+	+				
2013						+									
2073												+			
2134				+											+
2197							+				+				
2330														+	
2399				+											
2776														+	
2858			+						+			+			
3030															+
Sum	3	7	5	7	4	9	8	7	5	4	7	6	5	6	9

Conclusion

From the present study, it was deduced that using BA and NAA for the induction of root callus production caused an increase in the phenolic content compared with that of the main root. Decreasing the amount of NAA in callus 2 (MS + 0.5 mg/l BA + 0.1 mg/l NAA) resulted in a higher phenolic content than that in callus 1 (MS + 0.5 mg/l BA + 1 mg/l NAA). In addition, HPLC results for callus 2 show a marked increase in caffeic acid, coumarin, and luteolin content; however, the flavonoid content in the two calli decreased, and the metabolic profile of isoflavones showed great variation. DNA fingerprinting helps in the authentication and identification of *A. lappa* L., which is grown in Egypt. This is the first report on tissue culture and molecular biological study of this plant. The current literature, our previous work [9], and also the results of the present work prove the importance of the plant; hence, the authors recommended that the study on the effects of plant growth regulators, precursors, and other factors that increase the main active constituents of the plant, which can be used as a source of natural raw material for phytopharmaceuticals, be continued.

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Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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