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Original Paper

Comparative analysis of different methods of *Hedera helix* DNA extraction and molecular evidence of the functionality in PCR

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The most suitable method of total DNA extraction still remains the crucial step for many plant species, although there are many different protocols and commercial kits for DNA isolation. In this study, five different extraction protocols were analysed to find out the most appropriate method for DNA extraction from *Hedera helix* L. This species has numerous medical and pharmaceutical uses and is also characterized by antioxidant effects on human body. In spite of its wide medical utilization, it belongs to those plant species, where the genomic information is very limited. Comparing of different protocols resulted in the yield of extracted DNA that has ranged from 6.3 to 487 ng μl^{-1}. The purity of extracted DNA has ranged from 1.4 up to 2.0 A260/A280. All the extraction methods used in this study were evaluated not only in term of quantity and purity of DNA but also its functionality in the restriction endonuclease digestion and polymerase chain reaction based downstream analysis was performed.

Keywords: DNA extraction, CTAB, SDS,commercial kit, *Hedera helix* L.

1 Introduction

Hedera helix L. is polyploid complex of woody plant with evergreen leaves and with significant youth and adults developmental stages (McAllister, 1979, 1981; Rose, 1996; Metcalfe, 2005; Green et al., 2011). Ivy is intolerant to cold in winter (average temperature in the coldest month ≤-2 °C, Iversen, 1944) and this factor is likely limiting in determining the boundaries of its distribution in Northern and Eastern Europe (Metcalfe, 2005). It is very popular as a decorative plant and has many cultivars including non-climbing species. Because of evergreen and path-preferable feature, ivy is ideal for winter gardens and gardens design. Beside the ornamental utilization, most important are its medicinal properties. Ivy is known to contain saponins, that are the secondary metabolites known to have a number of pharmacological effects. It reduces cholesterol, has antibacterial, antifungal and antiviral influence (Bedir et al., 2000; Medeiros et al., 2002). Actually, ivy is widely used in pharmacy for its expectorant and antitussive effect.

Hedera helix L. is marked in literature as an example of genome size plasticity that occurs during typical developmental changes from juvenile to adult stage (Obermayer, 2000). When considering genomic point of view, only a very limited information exists about its genomic variability. Related to this, no standardized DNA extraction protocol is reported for ivy. For all the future genetic studies of ivy based on gene mapping, marker studies, phylogenetic analysis and population characterization, DNA extraction will be the crucial step.

During the plant DNA extraction specific problems arise that must be overcome. No universal extraction method was reported up to date, but a couple of methods become very popular in plant genetic and genomic studies. In principle they are based on lyses and purification with cetyl trimethylammonium bromide or using a dodecyl sulfate as detergent (Saghai-Maroof et al., 1984; Rogers and Bendich, 1985; Dellaporta et al., 1983; Jobes et al., 1995). DNA isolated only by the CTAB method (Valcárcel et al., 2002) and the commercial kit (Valcárcel et al., 2002; Clarke et al., 2006), are reported as to be used in the genetic analysis of ivy, and no comparative research of DNA extraction methods was reported yet.

The aim of the study was to compare two CTAB based, SDS based and two commercial DNA extraction methods according to the yield, purity and functionality of the extracted DNA for further genomic analysis of ivy.

2 Material and methods

2.1 Biological material and plant preparation

Juvenile healthy leaves of *Hedera helix* L. were collected *in situ* in different localities of Slovakia, Czech Republic,

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Poland and Croatia. In total 6 samples were used. They were treated immediatelly with etanol for the surface desinfection and stored under -20 °C until further processing. Subsequently, all of them were ground into a fine powder in liquid nitrogen by pestle and mortal, transfered into a 1,5 ml microcentrifuge tubes and stored until the DNA extraction.

2.2 DNA extraction procedure

In total, five extraction methods were followed – Dellaporta et al. (1983), Rogers and Bendich (1994), Padmalatha and Prasad (2006) and NucleoSpin Plant II – Macherey-Nagel and GeneJET Plant DNA purification commertial kits. The basic chemistry involved in the non-commercial individual tested methods were as follows.

Dellaporta et al. (1983) – extraction buffer: 100 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM 2-mercaptoethanol, additives: 20% SDS, 5 M potassium acetate, isopropanol, 50 mM Tris, 10 mM EDTA, pH 8.0, 3M sodium acetate and 80% ethanol.

Rogers and Bendich (1994) – 2× CTAB extraction buffer: 2% CTAB (w/v), 100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 1% PVP, chloroform/isoamyl alcohol (24:1), 10% CTAB (w/v), 0.7 M NaCl, CTAB precipitation buffer: 1% CTAB, 50 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, high salt TE buffer: 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 1 M NaCl, ethanol.

Padmalatha and Prasad (2006) – extraction buffer: 2% CTAB (w/v), 0.5 M Tris HCl pH 8.0, 0.5 M EDTA pH 8.0; 5.0 M NaCl, PVP (0.1 g 1 g^{-1} of leaf tissue, added while grinding); 3 M sodium acetate pH 5.2, 10 mg ml^{-1} ribonuclease A, chloroform/isoamylalcohol (24 : 1), phenol /chloroform/ isoamylalcohol (25 : 24 : 1), ethanol, TE buffer: 10 mM Tris HCl, 1 mM EDTA, pH 8.0. When commercial kits using, total genomic DNA was extracted following the manufacturers' instruction.

2.3 DNA analysis and PCR functionality testing

Quantity and quality of extracted DNA was analyzed by NanoPhotometer P-Class (Implen). The DNA functionality was tested by PCR amplification of iPBS markers. iPBS based length polymorphism analysis were performed in a total volume of 15 μl reaction volume containing 1× PCR buffer (ThermoScientific) 1.5 mmol l^{-1} MgCl2 (Invitrogen); 0.3 mmol l^{-1} each dNTP (Promega); 1U DreamTaq polymerase (ThermoScientific), 30 ng of template DNA and 800 nM iPBS primer 1867. Time and temperature profile of PCRs was as follows: 94 °C 2 min.; 35 cycles of 1 min. at 94 °C, 1 min. at 55 °C and 2 min. at 72 °C. Final extention was performed for 10 min. at 72 °C. PCR products were separated in 2% (w/v) agarose gels in 1 × TBE buffer. Gels were stained by GelRed™ and digitally photographed. All PCR amplifications were repeated at least twice to establish reproducibility.

3 Results and discussion

In this research, juvenile leaves of *Hedera helix* L. were harvested from *in situ* conditions and subjected to the different total genomic DNA extraction procedures to find the most suitable one for the application of retrotransposon based marker techniques. Ivy has high level of polyphenols that decreases the quality of extracted DNA. Because a high quality DNA is needed for retrotransposon based marker techniques, three standart protocols – Dellaporta et al. (1983), Rogers and Bendich (1994), Padmalatha and Prasad (2006) and two extraction kits – GeneJET™ Plant Genomic DNA Purification Mini Kit – ThermoScientific and NucleoSpin Plant II – Macherey-Nagel for DNA extraction, were tested. Extraction of plant DNA is problematic and must be firstly proved for every individual species when comparing it to DNA extraction from animal tissues, because of the rigid cell wall that surrounds the plant cells. DNA isolation from plant tissues requires participation of carbohydrates and enzymes, that ensure lysis of cell wall (Manen et al., 2005). The presence of polysacharides, polyphenols and other organic compounds may pose problem in DNA isolation process (Cota-Sánchez et al., 2006). Thus, mature plant tissues are not recommended for DNA extraction as they contain high concentrations of polysaccharides,

Table 1 Total genomic DNA concentration and purity by using commercial isolation kits

DNA extraction kit	GeneJET Plant Genomic DNA purification Mini Kit		NucleoSpin Plant II - Macherey-Nagel	
Sample	A260/280	Concentration (ng μl^{-1})	A260/280	Concentration (ng μl^{-1})
1	1.5	5.0	1.6	8.5
2	1.5	4.0	1.7	10.5
3	1.5	7.5	1.7	11
4	1.65	6.0	1.8	14
5	1.7	9.0	1.5	7.5
6	1.5	6.5	1.5	12.3

Table 2 Total genomic DNA concentration (ng μl^{-1}) and purity using DNA extraction methods reported in the literature

Method	Padmalatha and Prasad (2006)		Dellaporta et al. (1983)		Rogers and Bendich (1994)	
Sample	A260/280	Concentration	A260/280	Concentration	A260/280	Concentration
1	1.7	210	1.8	300	1.4	310
2	1.86	250	1.8	850	1.5	350
3	1.6	345	1.9	520	1.5	180
4	1.94	475	1.9	485	1.7	130
5	1.89	1200	1.9	400	1.5	260
6	1.88	525	1.9	370	1.9	145

Table 3 Comparison of results of DNA extraction procedures tested for *Hedera helix* L.

Method/Characteristics	A 260/280	Average DNA concentration (ng μl^{-1})
GeneJET Plant Genomic DNA purification Mini Kit	1.56	6.3
NucleoSpin Plant II	1.63	10.6
Padmalatha and Prasad (2006)	1.81	361
Dellaporta et al. (1983)	1.87	487
Rogers and Bendich (1994)	1.58	229

polyphenols, and other secondary metabolites (Dabo et al.,1993; Zhang et al., 2000). Therefore, the juvenile leaves were chosen for the analysis.

After DNA extraction using the GeneJET Plant Genomic DNA purification Mini Kit was obtained DNA with lower purity than was expected and the concentration of DNA has ranged from 4.0 up to 9.0 ng μl^{-1}. When using the NucleoSpin Plant II the purity of isolated DNA ranged from 1.5 up to 1.8 and the concentration ranged from 8,5 up to 14 ng μl^{-1} (table 1). Actually, many commercial isolation kits may be used for DNA isolation, and purification, but only a limited amount of sample (20–200 mg) can be processed using available isolation kits and the successful DNA isolation depends mainly on the type of plant material (Demeke and Jenkins, 2009).

DNA extraction according to Padmalatha and Prasad protocol (2006) showed the purity of isolated DNA in the range from 1.6 up to 1.94 and the obtained concetration ranged from 210 up to 1200 ng μl^{-1}. When using protocol according Dellaporta et al. (1983) the purity of extracted DNA was in a range from 1.8 to 1.9 and concetration from 300 to 850 ng μl^{-1}. DNA isolation based on Rogers and Bendich protocol (1994) obtained purity in the range from 1.7 to 1.9 and the DNA concentration from 130–350 ng μl^{-1} (table 2).

When comparing all tested extraction protocols, the highest average concentration of DNA was obtained by the protocol of Dellaporta et al. (1983) – 487 ng μl^{-1}, followed by extraction method according to Padmalatha and Prasad (2006) with the concentration of 361 ng μl^{-1}. The average values of DNA purity was in range of 1.56–1.87 (table 3).

To analyse the effectivity of the extracted DNA in enzymatic amplification of specific targets, iPBS markers were amplified using PCR. Most amplified retrotransposon loci were obtained for the NucleoSpin Plant II isolation kit method. The DNA samples isolated by Rogers and Bendich (1994) and Dellaporta et al. (1983) were not suitable for PCR amplification – they show only a pure amplification pattern of iPBS (figure 1).

The highest total number of amplified iPBS loci was achieved with the use of NucleoSpin Plant II isolation kit-74 amplified loci for six analyzed samples and very similar with GeneJET Plant Genomic DNA Purification Mini Kit, and Padmalatha and Prasad (2006) (figure 2).

The functionality of DNA extracted from plant tissues is the most important evaluation factor as this determines the suitability of an extraction method. For analysis where retrotransposon markers are used, good quality DNA (high molecular weight DNA free of RNA, protein and phenol contaminants) in a concentration range from 60–100 ng μl^{-1} is required (Kalendar et al., 2010; Trebichalský et al., 2013). The GeneJET Plant Genomic DNA Purification Mini Kit extraction procol was successfully applied for total genomic DNA isolation from young ivy leaves followed by analysis of different *Hedera helix* L. markers (Žiarovská et al., 2015). When extracting DNA from plants from field conditions, choosing the exctraction method is a crucial step that affects the rest

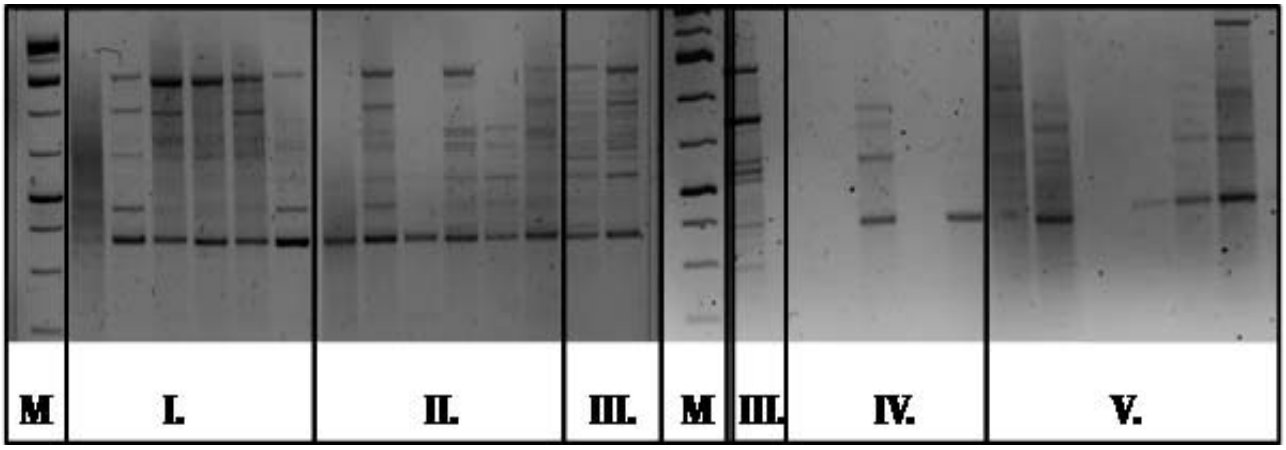

Figure 1 iPBS amplification pattern of *Hedera helix* L. genomic DNA isolated with teh use of different extraction methods. M-ladder, I. – GeneJET Plant Genomic DNA Purification Mini Kit, II. – Padmalatha and Prasad (2006), III.– NucleoSpin Plant II, IV. – Dellaporta et al. (1983), V. – Rogers and Bendich (1994)

of the analyses as many secondary metabolites are an inevitable part of plant tissues.

Biologically active compounds responsible for the medical use of *Hedera helix* L. are triterpene saponins (2.5–6%), bidesmosidic glycosides of hederagenin, hederacoside C (1.7–4.8%), hederacoside D (0.4–0.8%), hederacoside B (0.1–0.2%), and monodesmoside α-hederin (0.1–0.3%). Other groups of the identified biochemical compounds are represented by phenolics (flavonoids, anthocyanins, coumarins and phenolic acids), aminoacids, steroids, vitamins, volatile and fixed oils, β-lectins and polyacetylenes (Lutsenko et al., 2010). Plants also contain substances that have inhibitory effect in PCR, mainly, polysacharides and polyphenolic compounds. Polysacharides are difficult to separate from DNA during the extraction (Porebski et al., 1997; Lade et al., 2014) and in the subsequent PCR they interfere with polymerases. Moreover, phenol, alkaloids, polyphenols and polysacharides were identified to inhibit enzymes in the PCR or restriction reactions (Barra et al., 2012). Therefore, the first step in the work-flow of plant DNA analyses is to choose the most appropriate extraction method.

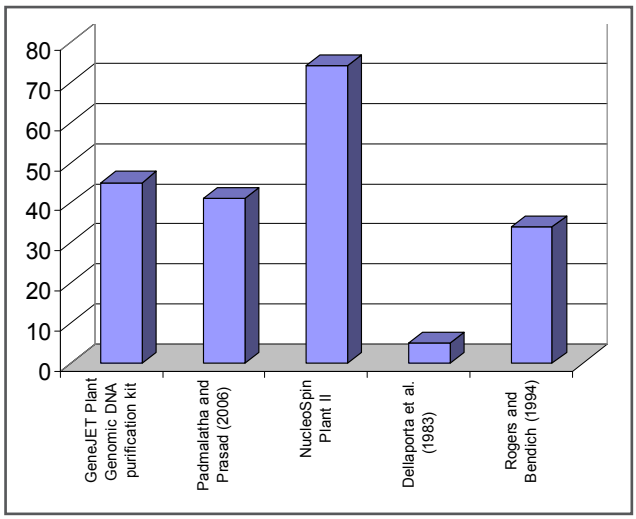

Figure 2 Total iPBS fragments amplified for the ivy samples using the individual methods tested in the study

Huaqiang et al. (2013) compared analysis of six DNA extraction methods in cowpea. They compared methods of Dellaporta, Saghai-Maroof, Rogers, Doyle, Aljanabi and commercial kit of E.Z.N.A. The highest yield of DNA was observed using the method of Aljanabi and Dellaporte. The lowest yield of exctracted DNA was reported for the method of Doyle. Comparing the DNA purity isolated according to Dellaporte, Saghai-Maroof, Rogers and Aljanabi protocols revealed that those methods are suitable, althouhg some RNA residues determinated by the elecrophoresis gel were still present. DNA isolated with the use of methods by Doyle and E.N.Z.A was relatively free from RNA and protein contamination.

Doosty et al. (2012) compared four different DNA extraction protocols in Satureja khuyistanica. They tested methods according to Dellaporta (1983), Doyle and Doyle (1990), Murry and Thompson (1980) and Kang and Yang (2004). Results obtained by the Dellaporta protocol showed low quality and quantity because SDS buffer used in this method interfered with the secondary metabolits. Also the results obtained according to the protocol by Doyle and Doyle and the protocol by Murry and Thompson had bad quality and quantity. The protocol by Kang and Yang was suitable for extracting DNA.

Sameer et al. (2009) compared different methods for DNA extraction from snap-frozen tissues to achieve good DNA quality and yield. They matched four manual DNA extraction methods – Phenol, 4M Sodium Chloride, 4M Ammonium Acetate, 4M Potassium Acetate with two commercial DNA isolation kits. They determined that all manual DNA extraction methods produced good yields

of DNA. The value of extracted DNA was 30 μg 100 μl^{-1} of TE. The value of DNA yield by using kits was above 14 μg 100 μl^{-1}. The purity of extracted DNA was for both methods in range from 1.67 to 2.04. The author claimed that better DNA extraction can be easily reached by salting than with phenol-chlorophorm or commercial kit.

Abu-Romman (2011) compared four plant DNA isolation methods of Bokszczanin and Prazybyla (2006), Doyle and Doyle (1987), Krizman et al. (2006) and Sarwat et al. (2006) in sage. The quality of DNA isolated by the Krizman method was better than the other three protocols. The amount of DNA isolated by Krizman protocol was 411 ng μl^{-1}, for Doyle and Doyle it was 178 ng μl^{-1}. The DNA yield by protocol of Sarwat was 277 μg μl^{-1} and by Bokszczanin and Prazybyla protocol was 205 μg μl^{-1}. Traditional CTAB method (Doyle and Doyle method) resulted in the lowest DNA yield and poor quality. The purity of DNA was better with Krizman method in value of 1.86.

4 Conclusions

In this study, five DNA extraction methods were compared to find the most appropriate one for the PCR based analysis of *Hedera helix* L. The yield of extracted DNA was in range of 6.3 up to 487 ng μl^{-1} in average and the purity of extracted DNA was not optimal for most of the tested extraction methods. Functionality of the extracted DNA was proved for the iPBS markers. Based on the results, the most suitable DNA extraction method for ivy PCR based analysis will be either commercial kits or Padmalatha and Prasad (2006) in spite of their worse purity characteristics.

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References

ABU-ROMMAN, S. (2011). Comparison of methods for isolating high quality DNA from sage (*Salvia officinalis*). *Journal of Medicinal Plants Research*, vol. 5, no. 6, pp. 938–941

BALANSARD, G. et al. (1980). Douvicidal and Antifungal Activities of α-Hederin Extracted from *Hedera helix* leaves. *Planta Medica*, vol. 39, p. 234.

BARRA, M. et al. (2012). Simple and robust DNA extraction mehod for the large-scale analysis of genotypes containing high polyphenolic content, such as landraces of *Solanum tuberosum* and *Zea mays*. *Plant breeding, genetic and genetic resources*, vol. 39, no. 3, pp. 593–601.

BEDIR, E. et al. (2000). Triterpene Saponins from the Fruits of *Hedera helix*. *Phytochemistry*, vol. 53, pp. 905–909.

CLARKE, M.M. et al. (2006). Prevalence of different horticultural taxa of ivy (*Hedera* spp., Araliaceae) in invading populations. *Biological Invasions*, vol. 8, pp. 149–157.

COTA-SÁNCHEZ, H. J. et al. (2006). Ready-to-Use DNA Extracted with a CTAB Method Adapted for Herbarium Specimens and Mucilaginous Plant Tissue. *Plant Molecular Biology Reporter*, vol. 24, no. 2, pp.161–167.

CRESPIN, F. et al. (1995). Identification of 3O-beta-D-Glucopyranosyl Hederagenin from the Leaves of *Hedera helix*, *Fitoterapia*, vol. 66, pp. 47.

DABO, S. M. et al. (1993). A method for the isolation of nuclear DNA from cotton (Gossypium) leaves. *Analytical Biochemistry,* vol. 210, pp. 34–38.

DELLAPORTA, S. L. et al. (1983). A Plant DNA Minipreparation: Version II. Plant Mol. Biol. Rep., vol. 1, pp. 19–21.

DEMEKE, T. and JENKINS, R. (2010). Influence of DNA extraction methods, PCR inhibitors and quantification methods on Real time PCR assay of biotechnology-derived traits. *Analytical and Bioanalytical Chemistry*, vol. 396, pp. 1977–1990.

DOOSTY, B. et al. (2012). Comparative Analysis and Optimization of Different DNA Extraction Protocols in Satureja khuzistanica. *International Journal of Biology*, vol. 4, no. 4, pp. 112–116.

ELIAS, R. et al. (1991). Triterpenoid saponins from the leaves of *Hedera helix*. *Journal of Natural Products*, vol. 54, pp. 98–103.

GREEN, A. F. et al. (2011). Phylogeny and Biogeography of Ivies (Hedera spp., Araliaceae), a Polyploid Complex of Woody Vines. *Systematic Botany*, vol 36, no. 4, pp. 1114–1127.

HUAQIANG, T. et al. (2013). Comparative Analysis of Six DNA Extraction Methods in Cowpea (*Vigna unguiculata* L. Walp). *Journal of Agricultural Science*, vol 5, pp. 82–90.

IVERSEN, J. (1944). Viscum, Hedera and Ilex as climatic indicators. Geologiska Föreningers *Stockholm Förhandlinger*, vol. 66, pp. 463–483.

JOBES, D. V. et al. (1995) Plant DNA isolation: A Method to Efficiently Remove Polyphenolics, Polysaccharides, and RNA. *Taxon*,vol. 44, pp. 379–386.

KALENDAR, R. et al. 2010. iPBS: a universal method for DNA Fingerprinting and retrotransposon isolation. *Theoretical and Applied Genetics*, vol. 121, pp. 1419–1430.

KRAJEWSKA, A. (1981). Phytochemical Studies of *Hedera helix* Herbage, Flowers and Fruits. *Herba Polonica*, vol. 27, pp. 303–309.

LADE, B.D. et al. (2014). Efficient genomic DNA extraction protocol from medicinal rich Passiflora foetida containing high level f polysacharide and polyphenol. *SpingerPlus*, vol. 3, p. 457.

LUTSENKO, Y. et al. (2010). *Hedera helix* as a medicinal plant. *Herba Polonica*, vol. 56, pp. 83–96.

MAJESTERSAVORNIN, B. et al. (1991). Saponins of the ivy plant. *Planta Medica*, vol. 57, p. 260–262.

MANEN, J. F. et al. (2005). A Fully Automatable Enzymatic Method for DNA Extraction from Plant Tissue. *BMC Plant Biology*, vol. 5, no. 23, pp. 1–9.

McALLISTER, H. A. (1979). The species of ivy. *Ivy Exchange Newsletter*, vol. 1, pp. 4–5.

McALLISTER, H. A. (1981). New work on ivies. International Dendrological Society Year Book, p. 106–109.

MEDEIROS, J.R. et al. (2002). Bioactive components of *Hedera helix*. Arquipélago, Life and Marine Sciences 19A, pp. 27–32.

METCALFE, D. J. (2005). *Hedera helix*. *Journal of Ecology: Biological flora of the British isles*, vol. 93, no. 240, pp. 632–648.

OBERMAYER, R. and GREILHUBER, J. (2000). Genome size in *Hedera helix* L. – a clarification. *Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics*, vol. 53, no. 1. doi: http://dx.doi.org/10.1080/00087114.2000.10589175

PADMALATHA K. and PRASAD M. N. V. (2006). Optimization of DNA isolationand PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India. *African Journal Biotechnology*, vol. 5, no.3, pp. 230–234.

POREBSKI et al. (1997). Modification of CTAB extraction protocol for plants containing high polysacharide and polyphenol components. *Plant Molecular Biology Reporter*, vol. 15, pp. 8–15.

RAO, G.S. et al. (1974). Antiviral Activity of Triterpenoid Saponins Containing Acylated β-Amyrin Aglycones. *Journal of Pharmaceutical Sciences*, vol. 63, pp. 471–473.

ROGERS, S. and BENDICH, A. (1994). Extraction of total cellular DNA from plants, algae and fungi. *Plant molecular biology manual*, vol. 8, no. 1, pp. 15–20.

ROSE, P. Q. (1996). The gardener's guide to growing ivies. London: David and Charles.

SAMEER, S., A. et al. (2009). Comparison of the Different Methods of DNA Extraction from Snap-frozen Tumor Tissues: Our Experience. *International Journal of Biotechnology and Biochemistry*, vol. 1, no. 5, pp. 43–50.

SAGHAI-MAROOF, M. A. et al. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *PNAS*, vol. 81, pp. 8014–8018.

SCHLOSSER, E. (1973). Role of Saponins in Antifungal Resistance. II. The Hederasaponins in Leaves of English Ivy. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz*, vol. 80, pp. 704–710.

TREBICHALSKÝ, A. et al. (2013). Detection of genetic relationships among spring and winter triticale (× Triticosecale witt.) and rye cultivars (*Secale cereale* L.) by using retrotransposon-based markers. *Czech Journal of Genetics and Plant Breeding*, vol. 49, pp. 171–174.

TRUTE, A. et al. (1997). *In vitro* antipasmodic compounds of the dry extract obtained from *Hedera helix*. *Planta Medica*, vol. 63, p. 125–129.

VALCÁRCEL, V. et al. (2003). Chloroplast and nuclear evidence for multiple origins of polyploids and diploids of Hedera (Araliaceae) in the Mediterranean basin. *Molecular Phylogenetics and Evolution*, vol. 27, pp. 1–20.

ZHANG, J. and STEWART, J.M. (2000). Economic and rapid method for extracting cotton genomic DNA. *Journal of Cotton Science*, vol. 4, pp. 193–201.

ŽIAROVSKÁ, J. et al. (2016). Utilization od different markers for *Hedera helix* L. germplasm evaluation. *Journal of Microbiology, Biotechnology and Food Sciences*, special vol. 1, pp. 23–26.

