



Rapid discrimination of the native medicinal plant *Adenostemma lavenia* from its adulterants using PCR-RFLP

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ABSTRACT

Background. In Taiwan, the aerial part of *Adenostemma lavenia* (Al) is used in the form of herbal tea or in a folk remedy primarily to mitigate inflammatory conditions in the lungs and liver. Due to the excellent health benefits of Al against inflammation, it has become increasingly crucial and in great demand during the COVID-19 pandemic. However, Al has been found to be adulterated with *Wedelia biflora*, *Sigesbeckia orientalis*, and/or *Wedelia chinensis* because of similarities in appearance and vernacular names.

Methods. This study aimed to develop a PCR-RFLP DNA molecular method for the authentication of Al. The restriction enzyme *Bsr*I was used according to the sequencing and alignment results of PCR products in the ITS2 regions of Al and its adulterants. Gel electrophoresis resulted in the clear separation of Al and its adulterants into two distinct categories.

Results. In conclusion, the PCR-RFLP authentication method developed herein provides an easy, rapid, and accurate method to distinguish Al from its adulterants to assure user health and safety.

Subjects Biotechnology, Food Science and Technology, Molecular Biology, Plant Science

Keywords *Adenostemma lavenia*, Identification of origin, Molecular identification, Functional food

INTRODUCTION

The medicinal plant *Adenostemma lavenia* (L.) Kuntze (Al) is known in Taiwan by its Chinese name “Xia-Tian-Ju (XTJ)” and grows widely in the tropical regions of Asia and the Pacific Islands (Cheng, Hufford & Doorenbos, 1979). The fruit of Al contains glands and mucus, which tends to stick to hands or clothes like glue, so Al is known colloquially in Chinese as “Ma-Zhi-Hu (MZH)”. Al is an herbaceous plant that is native to Taiwan and well cultivated in the cities of Yuanlin and Chiayi of Taiwan for applications in folk remedies and dietary supplements (Cheng, Hufford & Doorenbos, 1979; Lai et al., 2020).

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The aerial part of AI is used in the form of herbal tea or in a medicinal cuisine to mitigate inflammatory conditions in the lungs and liver and to treat skin wounds and ulcers (Cheng, Hufford & Doorenbos, 1979; Prasad & Shyma, 2013; Prasad, Shyma & Raghavendra, 2013). According to previous literature, the main secondary compounds of AI are monoterpenes, sesquiterpenoids, *ent*-kaurane-type diterpenoids, triterpenes, and steroids (Cheng, Hufford & Doorenbos, 1979; Lin, 2013; Shimizu et al., 1990; Yang et al., 2007). Several reports have shown that the health benefits of AI are associated with its bioactive components, mainly *ent*-kaurane-type diterpenoids. The *ent*-kaurane-type diterpenoids, such as adenostemmoic acid B and *ent*-11 α -hydroxy-15-oxo-kaur-16-en-19-oic acid, have been reported to be the most important bioactive compounds in AI, and they have been shown to possess physiological activities that include those against cancer, inflammation, aging, and melanogenesis (Batubara et al., 2020; Hamamoto et al., 2020; Maeda et al., 2021; Shimizu et al., 1990). Moreover, the ethyl acetate fractions of AI extract have been reported to significantly alleviate the inflammatory symptoms of pneumonia in lipopolysaccharide-induced lung injury (Chen et al., 2019). Thus, AI features valuable beneficial functions as well as the feasibility of being used as a functional food for the prevention of and/or therapy for inflammatory conditions (Hamamoto et al., 2020).

As an herbal tea or in a dietary supplement, AI has been claimed to be beneficial to lung health, so it has become increasingly important and in great demand during the COVID-19 pandemic in Taiwan (Lai et al., 2020). Based on our previous field investigation, we found that AI is sometimes adulterated with three other species in Asteraceae that are similar in appearance and/or have vernacular names similar to those of AI: *Wedelia biflora* (L.) DC. (Wb), *Sigesbeckia orientalis* L. (So), and *Wedelia chinensis* (Osbeck) Merr. (Wc) (Lai et al., 2020). Such adulteration may impair the food or medication safety, health-promoting abilities, and consumer confidence in AI.

Although the health-benefitting properties and chemical components of AI have already been scientifically reported, there have not yet been any relevant authentication or quality control research reports on AI published. Phytochemical component analysis and various traditional identification methods, such as organoleptic, macroscopic, and microscopic identification techniques, not only rely deeply on a researcher's expertise and experience but also cannot be applied alone to closely related species that possess similar chemical components and morphological structures (Han et al., 2016). Thus, DNA molecules are more desired and promising targets for developing an examination method for identifying herbal medicines. At present, a variety of DNA sequence-based species identification methods have been developed and applied, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Ha et al., 2002; Kaundun & Matsumoto, 2003). The aforementioned methods of DNA polymorphism analysis are species specific, cost-effective, rapid, and feasible for developing alternative assays to avoid adulteration (Abubakar et al., 2018; Yang et al., 2020; Zhu et al., 2018).

Adulteration of an herbal medicine can threaten the health of users (Han et al., 2016). To the best of our knowledge, there has not been a report that utilizes DNA molecular techniques to authenticate AI and separate it from its misused adulterants by means of

DNA molecular markers in the ITS region of the nuclear rDNA. Thus, this study aimed to develop a simple, reliable, and accurate PCR-RFLP DNA molecular method for AI market supervision to assure the health of users in terms of safety and efficacy of the medicine. Eventually, it is hoped that this PCR-RFLP authentication method for AI will be validated by commercially available AI market samples from throughout Taiwan.

MATERIALS & METHODS

Plant materials

To obtain the reference DNA sequences for AI and its adulterants, viz., Wb, So, and Wc, all plants were collected and authenticated with ten batches of AI, three batches of Wb, two batches of So, and two batches of Wc, respectively, at different localities in Taiwan (Fig. 1, Table 1). For the specific investigation of AI market samples, the fourteen AI market samples were randomly purchased from herbal shops around Taiwan by referring to AI as XTJ or using the vernacular name MZH. All herbal samples were authenticated by Professor Yuan-Shiun Chang (China Medical University), and they were deposited in the collection at the School of Pharmacy at China Medical University.

DNA extraction

Dried leaves from the authenticated samples of AI and its adulterants were collected and ground with liquid nitrogen into powder to allow for DNA extraction (Yang et al., 2020). Total DNA was isolated from each sample of homogenized plant tissue utilizing a Plant Genomic DNA Purification Kit (GeneMark, Atlanta, GA, USA) according to the manufacturer's instructions. After DNA extraction, the concentrations of total genomic DNA were determined by a spectrophotometer (NanoDrop-1000; Thermo Fisher Scientific, Waltham, MA, USA), and each sample was stored at -20°C .

Phylogenetic tree analysis

The sequence regions of internal transcribed spacer 2 (ITS2), Rubisco large subunit (*rbcL*), and transfer RNA asparagine-like (*trnL*) of AI and its adulterants were used for sequence alignment by Vector NTI Advanced 11 software, and three different phylogenetic trees were constructed by MEGA-X software (Lorusso & Gemmellaro, 2021). All the sequences of AI and its three adulterants were available and obtained from the Genbank database of the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>).

Polymerase chain reaction (PCR) and electrophoresis

First, the ITS2 regions of AI and its three adulterants were amplified by ITS-2F and ITS-3R primers using genomic DNA from various Asteraceous species as the template DNA (Yang et al., 2020). The two oligonucleotide primers ITS-2F (5'-ATGCGATACTTGGTGTGAAT-3') and ITS-3R (5'-GACGCTTCTCCAGACTACAAT-3') were designed and stemmed from the 18S and 26S rDNA sequences of AI and its three adulterants (Yang et al., 2020). DNA amplification was performed in a 25 μL reaction mixture including 12.5 μL *Taq* DNA polymerase 2 $\times$ Master Mix RED (Ampliqon A/S, Odense, Denmark), 9.5 μL double distilled water, 1 μL each of 10 μM ITS-2F and ITS-3R, and 1 μL template DNA, which was

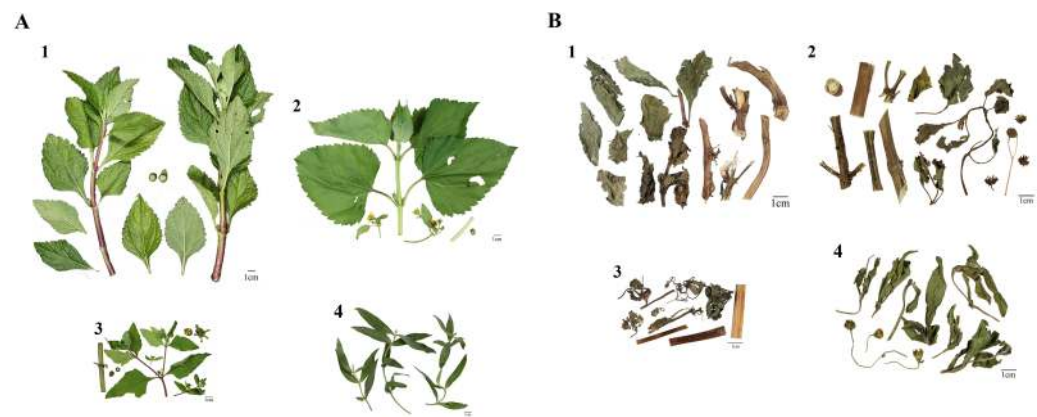


Figure 1 Morphological comparison of fresh plants (A) and dried herbal material (B) between *Adenostemma lavenia* (Al), *Wedelia biflora* (Wb), *Sigesbeckia orientalis* (So), and *Wedelia chinensis* (Wc). The numbers 1–4 represent Al, Wb, So, and Wc, respectively.
 [Full-size](#) [DOI: 10.7717/peerj.13924/fig-1](https://doi.org/10.7717/peerj.13924/fig-1)

Table 1 Information on samples of Al and its adulterants collected from various localities in this study.

Scientific name	Collection locations	Abbreviation	Voucher number
<i>Adenostemma lavenia</i>	Taipei	Al-1	CMURX2020002
	Taichung	Al-2	CMURX2020005
	Taichung	Al-3	CMURX2020006
	Taichung	Al-4	CMURX2020007
	Chiayi	Al-5	CMURX2020008
	Chiayi	Al-6	CMURX2020009
	Taichung	Al-7	CMURX2020010
	Hsinchu	Al-8	CMURX2020012
	Yunlin	Al-9	CMURX2020013
	Taichung	Al-10	CMURX2020014
<i>Wedelia biflora</i>	Hualian	Wb-1	CMURX2020001
	Taichung	Wb-2	CMURX2020015
	Changhua	Wb-3	CMURX2020016
<i>Sigesbeckia orientalis</i>	Changhua	So-1	CMURX2020017
	Taichung	So-2	CMURX2020018
<i>Wedelia chinensis</i>	Taichung	Wc-1	CMURX2020019
	Chiayi	Wc-2	CMURX2020020

diluted with double-distilled water until its concentration became one-tenth of its original value. The PCR conditions were 95 °C for 3 min; followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension for 5 min. The amplified products were resolved using 2.2% agarose gels that contained 0.1 µL/mL GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) in 1 × Tris-Acetate-EDTA buffer (TAE) with a 100-bp ladder as a marker.

PCR product sequencing and restriction fragment length polymorphism (RFLP) analysis

Each PCR product amplified by ITS-2F and ITS-3R was sequenced and aligned by Vector NTI Advanced 11 software, which was used to statistically analyze the percentage similarities of Al with its three adulterants. In addition, the sequences of Al and its adulterants were aligned by the Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) webserver (Sievers & Higgins, 2021). For these PCR products, sequence restriction maps were established and competed against one another on the NEBcutter V2.0 (<http://nc2.neb.com/NEBcutter2/>) webserver (Gargouri, Moalla & Kacem, 2021). One restriction enzyme, *Bsr*I, was selected and predicted to differentiate Al from the other three Asteraceous adulterants. Furthermore, we added a commercial vector, pcDNA3.1(+), as a positive control. The two oligonucleotide primers pcDNA-F (5'-CTGAATGAACTGCAGGACGA-3') and pcDNA-R (5'-GGCGGTGGAATCGAAATCTC-3') in the vector were designed to amplify a selected sequence containing a cutting site for *Bsr*I. Following the instructions from the manufacturer (New England Biolabs, Ipswich, USA), a 50 µL PCR-RFLP reaction mixture was prepared that contained 3 µL PCR product, 1 µL restriction enzyme, *Bsr*I, 41 µL double distilled water, and 5 µL NEBuffer. Next, all the mixtures, including those for Al and its three adulterants as well as the positive control, were incubated at 65 °C for 15 min. Finally, the RFLP products were determined using 2.2% agarose gels that contained 0.1 µL/ml GelRed nucleic acid gel stain (Biotium) in 1 × TAE with a 100-bp ladder as a marker.

Application of the PCR-RFLP assay for the survey of authenticity of *A. lavenia* from markets

Fourteen batches of commercial herbal samples of Al were collected as described above. DNA was extracted from each sample and determined by spectrophotometry. Then, PCR-RFLP was performed to discriminate between Al and its adulterants. The final results were obtained by DNA electrophoresis on agarose gels.

RESULTS

Morphological examination of plant specimens

Al and its three adulterants, Wb, So, and Wc, collected from various localities, were used, and their morphologies were compared, as illustrated in Fig. 1. Based on morphological appearance, the leaves and stems of Wc showed significant differences from those of Al, Wb, and So (panel 4 of Fig. 1A). There were few morphological differences in leaves or stems among Al, Wb, and So (panels 1, 2, and 3 of Fig. 1A, respectively). Moreover, the dried forms of Al, Wb, So, and Wc that were collected from various herbal shops showed no significant variation in appearance among them (panels 1, 2, 3, and 4 of Fig. 1B). Based on the morphological examination and comparison described above, Al is prone to be adulterated with other species similar in appearance, which alerts us to be concerned about the issue of Al adulteration with other species.

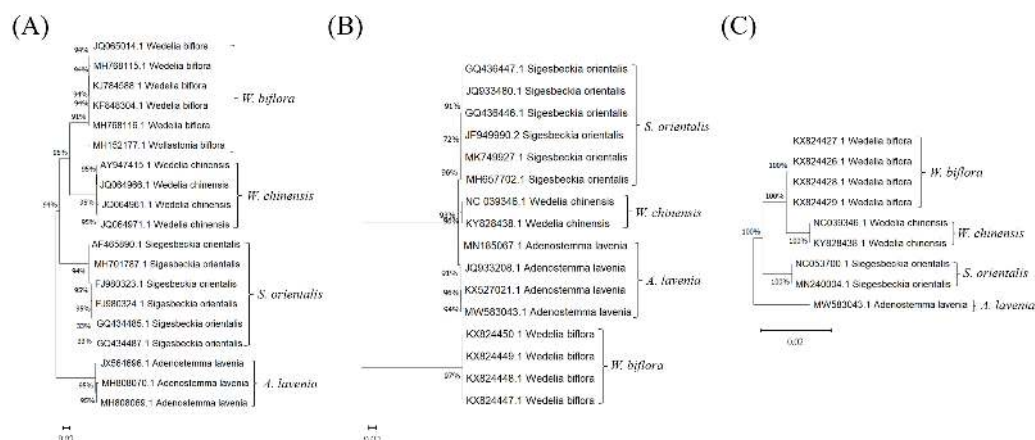


Figure 2 Phylogenetic trees of Al, Wb, So, and Wc produced for reference sequences in the (A) ITS2, (B) *rbcL*, and (C) *trnL* regions. The DNA sequences of different species were compared with the GenBank database of the National Center for Biotechnology Information (NCBI). The percentage on the node for each branch is a bootstrap score from 1,000 replicates.

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Phylogenetic tree analysis for DNA barcode selection

To select a suitable DNA barcode for the molecular authentication of Al and for distinguishing it from its adulterants, three commonly used potential DNA barcodes, ITS2, *rbcL* and *trnL*, were chosen to build phylogenetic trees to evaluate the sequences for their discriminatory power (Fig. 2). As illustrated in Fig. 2A, the phylogenetic analysis of the ITS2 regions was accomplished by the neighbor-joining (NJ) method. This ITS2-based phylogenetic analysis of Al and its adulterants formed four distinct clades (Fig. 2A). Furthermore, for *rbcL* and *trnL*, the phylogenetic trees produced also exhibited a similar discriminatory pattern in forming distinct clades for Al and its adulterants (Figs. 2B & 2C). These results indicate that ITS2, *rbcL*, and *trnL* could all be used for Al authentication. However, regarding the comparison among the genetic distances in ITS2-, *rbcL*- and *trnL*-based phylogenetic trees, the ITS2-based phylogenetic tree demonstrated greater genetic distances between the species than did the *rbcL*- and *trnL*-based phylogenetic trees. In further examining the sequence divergence in the ITS2 tree, 85–94% sequence similarities were found between Al and its three adulterants (Fig. 3 and Table 2). This is reflected in the sequence divergence in the ITS2 tree, which ranged from 6–15% between Al and its three adulterants. This strongly supports the credibility of this NJ tree, and the range implies that Al shows sufficient sequence divergence on the ITS2 tree, which increases the possibility of successful Al authentication once a rapid identification assay is established. In summation, ITS2 can be concluded to hold higher potential as a molecular marker than do *rbcL* or *trnL* to authenticate Al and distinguish it from its adulterants.

Development of a PCR-RFLP assay for *A. lavenia* authentication

Because it was concluded that ITS2 is a useful molecular marker for authenticating Al, ITS2 was herein used as a specific DNA marker for the development of a PCR-RFLP assay for the convenient authentication of Al plants. As illustrated in Fig. 4A, PCR amplified ITS2

A1	-----CTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTT	51
Wb	-----CGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTT	50
So	TGCGAATACTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTT	60
Wc	-GCGATTACTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTT	59

A1	GCGCCGGAAGCCTCTCGGTGAGGGCACGTCGCTGGGCGTCAACGATCACTGCGCCA	111
Wb	GCGCCGGAAGCCT-CTGGCTGAGGGCACGTCGCTGGGCGTCAACGATCACTGCGCCC	109
So	GCGCCTGAAGCCATCCGTTGAGGGCACGTCGCTGGGCGTCAACGATCACTGCGCCC	120
Wc	GCGCCGGAAGCCTTTTGGCTGAGGGCACGTCGCTGGGCGTCAACGATCACTGCGCCC	119
***** ** *****		
A1	CACCAAAATCCTTCATTGGATCAAGAA----TGTTGGGCGGAAGCTGGTCTCCTGTGCC	166
Wb	CACCAA--CCATCCCTGCCAGGGATGTGTGGGCTGGGCGGAGATTGGTCTCCCGTGCC	167
So	CACCAACCGTCCCTGCACGG--GAGC-TGTTGGACGGGCGGAGATTGGTCTCCCGTCA	177
Wc	CGCAAAACCATCCATACGAGGGATGTGTGGGCTGGGCGGAGATTGGTCTCCCGTGCC	179
* *		
A1	CATGGTGGGTTGGCCTAAATTC-GAGTCTGCTTAAGAGTGACGACACTGGTGGT	225
Wb	CATGGCGTGGTTGGCCTAAATA-GGAGTCCCTCAGGAGAGACGACGGCTAGTGGTGGT	226
So	TGTTGTGGGTTGGCCTAAATAGGAGC--CTCCAAAGGGTACGCAAGCTAGTGGTGGT	235
Wc	CATGGCGTGGTTGGCCTAAATAAGGAGTCTCCTCAGGAGAGACGACGACTAGTGGTGGT	239
* *		
A1	TGATTACACAGTGTCTCGTGTGCTGCTTTGATCTTCAAGGGAGAACTCTTAAAGTA	285
Wb	TGATAAGACAGTGTCTCGCGCGTGGGTTTGTAGTCTTGAGGGGATACTCTTGAAGTAA	286
So	TGATAACACAGTGTCTCGTGTGCTGCTTTGATCCTTGGGGAGGA-ACTC-TTGAATA	293
Wc	TGATAAGACTGTGGCTCGCGTGTGCTTTGTAGTCTCGAGGGGATACTCTTAAACTAC	299
**** ** ***** * * * * * * * * * * * * * * * * * *		
A1	CCCTTATGTGCTGTCTTTTGGTAGCTCTTGGATTGCGACCCAGGTCAGGCGGGACTACC	345
Wb	CCCAACGTGTGTCTTGACGATGATGCTTGGATCGCGACCCAGGTCAGGCGGGACTACC	346
So	CCCGTGTGTGTGTCTTTTGTATGATGCTTGGATCGCGACCCAGGTCAGGCGGGACTACC	353
Wc	CC-GGGTGGTGTGTCTTGTATGACGCTTGGATCGCGACCCAGGTCAGGCGGGACTACC	358
** *		
A1	CGCTGAGTTTAAGCATATCAATAAGCGAGGAAAAGAACTTACAAGGATTCCCTTAGTA	405
Wb	CGCTGAGTTTAAGCATATCAATAAGCGAGGAAAAGAACTTACAAGGATTCCCTTAGTA	406
So	CGCTGAGTTTAAGCATATCAATAAGCGAGGAAAAGAACTTACAAGGATTCCCTTAGTA	413
Wc	CGCTGAGTTTAAGCATATCAATAAGCGAGGAAAAGAACTTACAAGGATTCCCTTAGTA	418

A1	ACGGCGAGCGAACCGGGAATAGCCAGCTTGAAAATCGGATGGCTTGGTGTGTGAATTG	465
Wb	ACGGCGAGCGAACCGGGAACAGCCAGCTTGAAAATCGGTGGCTTGGTGTGTGAATTG	466
So	ACGGCGAGCGAACCGGGAACAGCCAGCTTGAAAATCGGACGGCTTGGTGTGTGAATTG	473
Wc	ACGGCGAGCGAACCGGGAACAGCCAGCTTGAAAATCGGTGGCTTGGTGTGTGAATTG	478

A1	TAGTCTG-----	472
Wb	TAGTCTA-----	473
So	TAGTCTGAAAAG----	486
Wc	TAGTCTGAGAAGCGTCA	496

Figure 3 ITS2 sequence comparison between A1 and its adulterants. The *Bsr* I recognition site is marked with a red box. An asterisk (*) represents an aligned nucleotide that is identical in all sequences. A hyphen (-) indicates a gap in the aligned sequence.

Full-size  DOI: 10.7717/peerj.13924/fig-3

Table 2 Analysis of sequence similarities for ITS2 regions between Al and its adulterants.

Species	Al	Wb	So	Wc
Al		85%	86%	86%
Wb			90%	88%
So				94%
Wc				

fragments, which were successfully produced and sequenced using ITS2 universal primers when the genomic DNA of Al, Wb, So, and Wc was used as templates. After sequencing, four different DNA sizes, 472 bp, 473 bp, 486 bp and 496 bp, which corresponded to Al, Wb, So and Wc, respectively, were resolved in agarose gel (Fig. 4A & Table 3). Among these, various ITS2 sequences showed a similar pattern of DNA size. It was difficult to distinguish Al from its adulterants instantly, but this could be accomplished by polymerase chain reaction (Fig. 4A). After predicting the restriction profiles for ITS2 by NEBcutter (<http://nc2.neb.com/NEBcutter2/>), only one restriction enzyme cutting site, *BsrI*, was recognized in Al, So, and Wc (Fig. 3). There were no *BsrI* cutting sites in ITS2 of Wb (Fig. 3). Thus, when *BsrI* was used to digest ITS2 from Al, Wb, So, and Wc, two DNA fragments (253 bp and 219 bp) produced from the Al sample, two fragments (472 bp and 14 bp) produced in the So, and two fragments (483 bp and 13 bp) produced in the Wc were revealed, which made it easy to discriminate Al and its adulterants into two distinct patterns (Fig. 4B & Table 3). It is worth noting that the restriction profiles with *BsrI* of the three adulterants, Wb, So, and Wc, demonstrated no significant difference in DNA banding patterns after electrophoresis (Fig. 4B, lanes 2–4). The ITS2 DNA from Wb was not affected by *BsrI* digestion. Simultaneously, for the validity of digestion, we chose a 700-bp sequence containing a cutting site for *BsrI* from a commercial vector, pcDNA3.1(+), to provide a positive control for the experiment. The result showed two DNA fragments (600 bp and 100 bp) for DNA banding patterns after electrophoresis. This result effectively demonstrated that the restriction enzyme was fully functioning. Furthermore, the typical RFLP pattern of Al was altered when the Al sample was mixed with one of three adulterants (Fig. 4C). Taken together, through the experimental investigation of restriction profiles, a PCR-RFLP analysis for Al authentication was established. This analysis could rapidly authenticate Al and discriminate its adulterants easily when adulteration of Al had occurred.

Application of PCR-RFLP assays for authenticated Al and its adulterants in different localities

To assure the applicability of the PCR-RFLP technique for DNA molecular identification of Al, ten authenticated Al and seven adulterated samples, including Wb, So, and Wc samples collected from various localities, were tested. As shown in Fig. 5, the ITS2 sequences of all the samples were amplified by PCR (Figs. 5A & 5B), and the DNA sequence lengths of the PCR-amplified ITS2 were expected to be the same as those in Fig. 4A. After *BsrI* digestion, all of the samples were cleaved, and a typical RFLP pattern for Al and Al adulterants was produced. These data suggest that the PCR-RFLP method could be applied to Al sample identification in various localities throughout Taiwan.

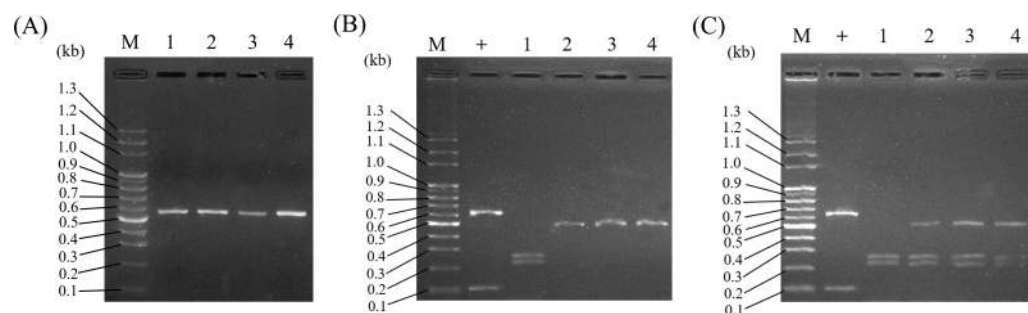


Figure 4 Establishment of PCR-RFLP pattern for AI authentication and discrimination of AI and its adulterants. (A) PCR-amplified ITS2 was revealed for the species AI, Wb, So, and Wc. Lane M, DNA marker; lanes 1–4, AI, Wb, So, and Wc, respectively. (B) PCR-RFLP patterns for AI authentication were produced in agarose gel. PCR-amplified ITS2 was digested by *Bsr* I. Lane M, DNA marker; +, positive control; lanes 1–4, AI, Wb, So, and Wc, respectively. (C) PCR-RFLP analysis for AI authentication and adulteration. Samples of AI mixed with its adulterants were examined by RFLP. Lane M, DNA marker; +, positive control; lane 1, AI; lanes 2–4, AI mixed with Wb, So, and Wc, respectively.

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Table 3 The DNA sequence lengths of predicted restriction fragments cleaved from the PCR-amplified ITS2 regions of AI and its adulterants.

Scientific name	Length (bp)	
	ITS2 ^a	Cleaved ITS2 ^b
<i>Adenostemma lavenia</i>	472	253/219
<i>Wedelia biflora</i>	473	473
<i>Sigesbeckia orientalis</i>	486	472/14
<i>Wedelia chinensis</i>	496	483/13

Notes.

^aThe lengths of ITS2 region of *Adenostemma lavenia* and its three adulterants were amplified by ITS-2F and ITS-3R primers.

^bPredicted restriction fragments of different sizes after *Bsr* I digested.

Application of the PCR-RFLP assay for the investigation of adulterants in AI samples from herbal markets

To investigate AI adulteration, commercial AI herbal plants purchased from various herbal markets were used for authentication and identification by the PCR-RFLP assay. There were a total of fourteen AI samples. As expected, the identification of the market samples clearly separated AI and its adulterants into two different categories. Among the fourteen commercially purchased AI samples, most were certified as authentic samples of AI by PCR-RFLP. There were three of the samples claimed to be AI that were actually adulterated (lanes 5 and 7 in Fig. 6A, lane 7 in Fig. 6B). The authentic samples of AI demonstrated DNA fragments of lengths 253 bp and 219 bp in a typical AI RFLP pattern. On the other hand, the adulterants' patterns are shown in lanes 5 and 7 in Fig. 6A and lane 7 in Fig. 6B. These results indicate that 21.4% (3/14) of the AI samples purchased randomly in herbal markets were adulterated. In summary, this established PCR-RFLP assay can indeed be applied for AI identification, and our AI authentication results showed that approximately 21.4% of the samples from herbal markets were adulterated.

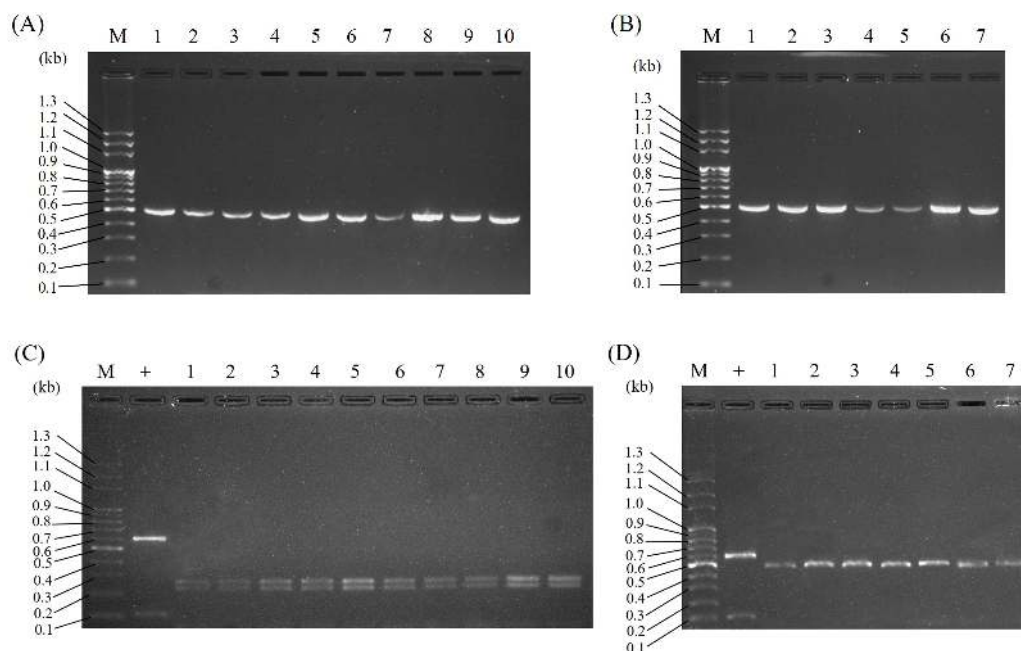


Figure 5 Application of PCR-RFLP analysis for the authentication of AI and its adulterants collected from various localities. The ITS2 regions of the four species AI, Wb, So, and Wc were amplified by PCR (A, B). RFLP analysis was conducted by *Bsr* I following PCR to differentiate AI and its adulterants (C, D). In panels (A) and (C), lane M indicates a DNA marker, + indicates a positive control, and lanes 1–10 indicate AI samples collected from various localities. For panels (B) and (D), lane M indicates a DNA marker, + indicates a positive control, lanes 1–3 indicate Wb samples collected from three different localities, lanes 4–5 indicate So samples collected from two different localities, and lanes 6–7 indicate Wc samples collected from two different localities.

Full-size [DOI: 10.7717/peerj.13924/fig-5](https://doi.org/10.7717/peerj.13924/fig-5)

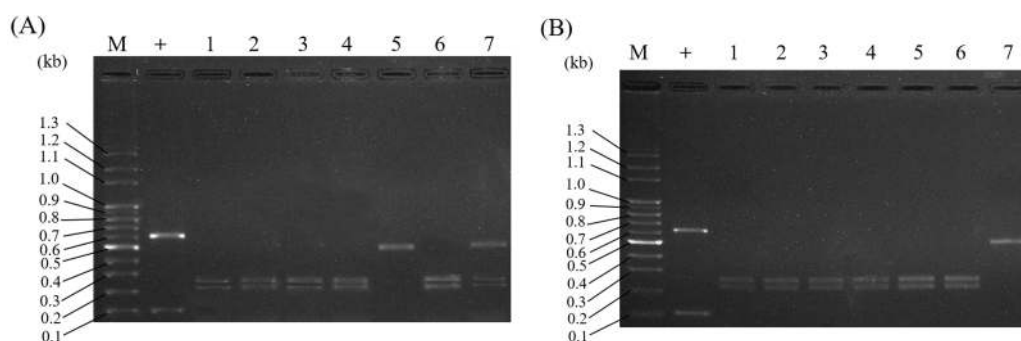


Figure 6 Application of PCR-RFLP analysis for the investigation of AI adulteration in AI samples from herbal markets. A total of fourteen commercial AI samples were collected from various herbal markets in Taiwan. ITS2 regions of all samples were subjected to RFLP analysis. The collected AI samples in (A) and (B) represent the samples purchased from different herbal vendors. Lane M in (A) and (B) indicates the DNA marker; + in (A) and (B) indicates the positive control.

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DISCUSSION

Differentiating AI from its adulterants is a critical issue because in Taiwan, AI is not only a native cultivated plant but also plays a crucial role in herbal tea, possessing value for lung protection and treatment (Cheng, Hufford & Doorenbos, 1979; Lin, 2013). Furthermore, the bioactive diterpene ingredients of AI have significant anti-inflammatory effects in pneumonia that may result from COVID-19 (Chen et al., 2019; Maeda et al., 2021). With the aforementioned health-promoting abilities of AI in mind, establishing an accurate appraisal of the authenticity of AI is essential. In addition, it should be noted that AI in Japan is included on the Red List as an endangered species, while AI in Taiwan is well cultivated (Maeda et al., 2021). This places even greater emphasis on the importance of discriminating between AI and its adulterants.

Among the variety of authentication methods, PCR-RFLP is a useful and popular tool for nonprofessional users, viz., traditional drug producers and managers, customs officers, and forensic specialists (Pennisi, 2007; Xin et al., 2015). Factors influencing a successful DNA molecular method include universal priming sites, a high level of amplification, an excellent DNA sequencing result, and enough disparity to identify the sample(s) (Parvathy et al., 2015). One of the key points of this study was to find suitable DNA loci to discriminate AI from three other species of plants often confused with it. In the present study, a phylogenetic evolutionary tree was built for AI and its adulterants using ITS2, *rbcL*, and *trnL* for comparison. The best one was found to be ITS2, which was supported by the values of nodes on almost all exceeding 90%, indicating a reliable evolutionary relationship (Alaklabi et al., 2021). Moreover, in previous research (Chen et al., 2010; Hollingsworth, Graham & Little, 2011; Xiong et al., 2016), ITS2 has been shown to be the best locus among seven different candidate barcodes (*psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, ITS2, and ITS) from medicinal plant species. ITS2 succeeded in the identification of species with 92.7% accuracy. Therefore, we chose the ITS2 region to serve as the main barcode for distinguishing AI from its adulterants.

This study was the first report to develop a PCR-RFLP assay for identifying AI and its adulterants as well as distinguishing between them by means of DNA barcoding. In past reports, research concerning the molecular biology of AI has been rare (Xia & Li, 2021). All four species used in this study have previously been detected to acquire their composition and the certain active ingredients they contain for the promotion of good health (Gao et al., 2018; Hamamoto et al., 2020; Shimizu et al., 1990; Yang et al., 2007). Some past studies have also utilized genomic ribosomal DNA sequences to differentiate interspecific relationships and build NJ trees with other plants (Gao et al., 2018). However, no one thus far has completed an in-depth exploration of a rapid and easy method to judge the authenticity of AI samples. Direct DNA sequencing is accurate in analyzing AI and the species with which it is confused. However, this technique is inefficient and time-consuming (Lin & Hwang, 2007). In contrast, the PCR-RFLP method used in this study is much more cost-effective, simple, and convenient, provided there is sufficient quantity and quality of DNA products for species identification. Furthermore, according to the results of this study, PCR-RFLP is appropriate for authenticating commercial samples of AI and its adulterants because

the DNA barcodes found apparently demonstrated its discriminatory power ([Ghatak, Muthukumaran & Nachimuthu, 2013](#)). Nevertheless, this method does have a restriction in that small fragments would hardly be seen owing to the insensitivity of gel electrophoresis. In general, fragments smaller than 80 bp are difficult to identify on agarose gels ([Wolf, Rentsch & Hübner, 1999](#)). Although this disadvantage exists, in this study, we could still clearly detect the differences between AI and its adulterants.

With a certain testing method of quality control for AI needed, we found that PCR-RFLP can be widely used to investigate the authenticity of the AI found in herb markets all over Taiwan. On the other hand, in addition to the development of the PCR-RFLP method, we also performed an interesting modified experiment with those AI samples containing adulterants, which may include two or more species. In our study, with the same identification material, the pure AI samples could still be easily discriminated from the impure. The adulterated mixtures were also clearly detected because of the pattern of three fragments of different sizes. This phenomenon reaffirms the validity of the PCR-RFLP method for determining the purity of AI.

Toward the end of the period when we were using the material for the discrimination of AI from its potential adulterants, we randomly collected fourteen commercial samples of AI from herbal shops in various localities in Taiwan. Through the evidence obtained from the PCR-RFLP method, our results demonstrated that most of these samples were real AI; nevertheless, there were three samples that contained adulterants. Among these three, two consisted of other plants that had been substituted for AI, and one of them contained AI mixed with another plant. This phenomenon represents the necessity of the development of a rapid method for identification of authentic AI.

In the research on commercial herbal samples of AI, we successfully identified AI and its adulterants in fourteen collected market AI samples. The results of our examination indicate that PCR-RFLP is useful for DNA sequence analysis and can easily and precisely discriminate between AI and its adulterants. However, the disappearance of small DNA fragments was an imperfect aspect of this experiment. This problem may be addressed by the technique of capillary electrophoresis (CE). With typical CE, the detection of DNA fragments will be more accurate and provide better patterns for identification. In brief, in terms of the reports that AI is a threatened species in some places ([Maeda et al., 2021](#)), economical constraints and the difficulty of obtaining it may incentivize traders to replace it with cheaper substitutions and sell the adulterated product as if it were AI ([Kool et al., 2012](#); [Xin et al., 2015](#)). Moreover, with the domestic food and drug safety situation gradually becoming more stringent, manufacturers and individuals have turned their attention from substitution to “illegal addition” and “process control” ([Siddiqui et al., 2021](#); [Xin et al., 2015](#)). For this reason, raw material quality control has become a kind of security assurance for society. On the other hand, AI plays a vital role in the herbal market of Taiwan. However, the lack of ability to verify the authenticity of AI has been a vital issue.

CONCLUSIONS

This work utilized the method of PCR-RFLP to discriminate samples of AI to determine which were pure and which were adulterated. With the PCR-RFLP method, an excellent

investigatory tool is available. Finally, this study contributes to related research regarding AI and quality control as well as the security of consumer health.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Kunchang Wu conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Yunchen Liu performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Bocheng Yang performed the experiments, prepared figures and/or tables, and approved the final draft.
- Yenyong Kung conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Kaiwei Chang conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Mengshiou Lee conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The ITS2 DNA sequences of *Adenostemma lavenia*, *Wedelia biflora*, *Sigesbeckia orientalis*, and *Wedelia chinensis* described here are available at GenBank: [OM095431.1](#), [OM095434.1](#), [OM095435.1](#), and [OM095436.1](#).

Data Availability

The following information was supplied regarding data availability:

All the raw data of ITS2 sequences of *Adenostemma lavenia* and its three adulterants viz *Wedelia biflora* (L.) DC., *Sigesbeckia orientalis* L., and *Wedelia chinensis* (Osbeck) Merr. are available in the [Supplementary File](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.13924#supplemental-information>.

REFERENCES

- Abubakar BM, Salleh FM, Omar MSShamsir, Wagiran A. 2018. Assessing product adulteration of *Eurycoma longifolia* (Tongkat Ali) herbal medicinal product using DNA barcoding and HPLC analysis. *Pharmaceutical Biology* 56:368–377 DOI 10.1080/13880209.2018.1479869.
- Alaklabi A, Ahamed A, Qthanin RNAl, Arif IA, Panneerselvam A, Al-Khulaidi AW. 2021. Molecular characterization of endangered endemic plant *Aloe pseudorubroviolacea* using chloroplast *matK* and plastid *rbcL* gene. *Saudi Journal of Biological Sciences* 28:1123–1127 DOI 10.1016/j.sjbs.2020.11.042.
- Batubara I, Astuti RI, Prastya ME, Ilmiawati A, Maeda M, Suzuki M, Hamamoto A, Takemori H. 2020. The antiaging effect of active fractions and *ent*-11 α -hydroxy-15-oxo-kaur-16-en-19-oic acid isolated from *Adenostemma lavenia* (L.) O. Kuntze at the cellular level. *Antioxidants* 9(8):719 DOI 10.3390/antiox9080719.
- Chen JJ, Deng JS, Huang CC, Li PY, Liang YC, Chou CY, Huang GJ. 2019. p-coumaric-acid-containing *Adenostemma lavenia* ameliorates acute lung injury by activating AMPK/Nrf2/HO-1 signaling and improving the anti-oxidant response. *The American Journal of Chinese Medicine* 47:1483–1506 DOI 10.1142/s0192415x19500769.
- Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X, Luo K, Li Y, Li X, Jia X, Lin Y, Leon C. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLOS ONE* 5:e8613 DOI 10.1371/journal.pone.0008613.
- Cheng PC, Hufford CD, Doorenbos NJ. 1979. Isolation of 11-hydroxyated kauranic acids from *Adenostemma lavenia*. *Journal of Natural Products* 42:183–186 DOI 10.1021/np50002a009.
- Gao X, Wei J, Hong L, Fan S, Hu G, Jia J. 2018. Comparative analysis of chemical composition, anti-Inflammatory activity and antitumor activity in essential oils from *Siegesbeckia orientalis*, *S. glabrescens* and *S. pubescens* with an ITS sequence analysis. *Molecules* 23(9):2185 DOI 10.3390/molecules23092185.
- Gargouri H, Moalla N, Kacem HH. 2021. PCR-RFLP and species-specific PCR efficiency for the identification of adulteries in meat and meat products. *European Food Research and Technology* 247:2183–2192 DOI 10.1007/s00217-021-03778-y.
- Ghatak S, Muthukumaran RB, Nachimuthu SK. 2013. A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. *Journal of Biomolecular Techniques* 24:224–231 DOI 10.7171/jbt.13-2404-001.
- Ha WY, Shaw PC, Liu J, Yau FC, Wang J. 2002. Authentication of *Panax ginseng* and *Panax quinquefolius* using amplified fragment length polymorphism (AFLP) and directed amplification of minisatellite region DNA (DAMD). *Journal of Agricultural and Food Chemistry* 50:1871–1875 DOI 10.1021/jf011365l.

- Hamamoto A, Isogai R, Maeda M, Hayazaki M, Horiyama E, Takashima S, Koketsu M, Takemori H. 2020. The high content of *ent*-11 α -hydroxy-15-oxo-kaur-16-en-19-oic acid in *Adenostemma lavenia* (L.) O. Kuntze leaf extract: with preliminary in vivo assays. *Foods* 9:73 DOI 10.3390/foods9010073.
- Han J, Pang X, Liao B, Yao H, Song J, Chen S. 2016. An authenticity survey of herbal medicines from markets in China using DNA barcoding. *Scientific Reports* 6:18723 DOI 10.1038/srep18723.
- Hollingsworth PM, Graham SW, Little DP. 2011. Choosing and using a plant DNA barcode. *PLOS ONE* 6:e19254 DOI 10.1371/journal.pone.0019254.
- Kaundun SS, Matsumoto S. 2003. Identification of processed Japanese green tea based on polymorphisms generated by STS-RFLP analysis. *Journal of Agricultural and Food Chemistry* 51:1765–1770 DOI 10.1021/jf020821i.
- Kool A, De Boer HJ, Krüger A, Rydberg A, Abbad A, Björk L, Martin G. 2012. Molecular identification of commercialized medicinal plants in southern Morocco. *PLOS ONE* 7:e39459–e39459 DOI 10.1371/journal.pone.0039459.
- Lai SS, Lin TH, Hung YY, Chang FP, Chao SW, Wu KC. 2020. Investigation and authentication of *Adenostemma lavenia* (L.) Kuntze (Ma Zhi Hu) sold in Taiwan. In: *35th symposium on natural products. Taiwan*.
- Lin HT. 2013. The constituents of the *Adenostemma lavenia* (L.) O.Kuntze. Master Thesis, Southern Taiwan University of Science and Technology.
- Lin WF, Hwang DF. 2007. Application of PCR-RFLP analysis on species identification of canned tuna. *Food Control* 18:1050–1057 DOI 10.1016/j.foodcont.2006.07.001.
- Lorusso N, Gemmellaro D. 2021. Applying phylogenetic tree building in MEGA X to forensic applications for identifying unknown specimens.
- Maeda M, Suzuki M, Fuchino H, Tanaka N, Kobayashi T, Isogai R, Batubara I, Iswanti D, Matsuno M, Kawahara N, Koketsu M, Hamamoto A, Takemori H. 2021. Diversity of *Adenostemma lavenia*, multi-potential herbs, and its kaurenoic acid composition between Japan and Taiwan. *Journal of Natural Medicines* 76(1):132–143 DOI 10.1007/s11418-021-01565-3.
- Parvathy VA, Swetha VP, Sheeja TE, Sasikumar B. 2015. Detection of plant-based adulterants in turmeric powder using DNA barcoding. *Pharmaceutical Biology* 53:1774–1779 DOI 10.3109/13880209.2015.1005756.
- Pennisi E. 2007. Taxonomy. Wanted: a barcode for plants. *Science* 318:190–191 DOI 10.1126/science.318.5848.190.
- Prasad AGD, Shyma TB. 2013. Medicinal plants used by the tribes of Vythiri taluk, Wayanad district (Kerala state) for the treatment of human and domestic animal ailments. *Journal of Medicinal Plants Research* 7:1439–1451.
- Prasad AGD, Shyma TB, Raghavendra MP. 2013. Plants used by the tribes for the treatment of digestive system disorders in Wayanad district, Kerala. *Journal of Applied Pharmaceutical Science* 3:171–175 DOI 10.7324/JAPS.2013.3830.
- Shimizu S, Miyase T, Umehara K, Ueno A. 1990. Kaurane-type diterpenes from *Adenostemma lavenia* O Kuntze. *Chemical & Pharmaceutical Bulletin* 38:1308–1312 DOI 10.1248/cpb.38.1308.

- Siddiqui MA, Khir MHM, Witjaksono G, Ghumman ASM, Junaid M, Magsi SA, Saboor A. 2021.** Multivariate analysis coupled with M-SVM classification for lard adulteration detection in meat mixtures of beef, lamb, and chicken using FTIR spectroscopy. *Foods* **10**:2405 DOI [10.3390/foods10102405](https://doi.org/10.3390/foods10102405).
- Sievers F, Higgins DG. 2021.** The clustal omega multiple alignment package. *Methods in Molecular Biology* **2231**:3–16 DOI [10.1007/978-1-0716-1036-7_1](https://doi.org/10.1007/978-1-0716-1036-7_1).
- Wolf C, Rentsch J, Hübner P. 1999.** PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. *Journal of Agricultural and Food Chemistry* **47**:1350–1355 DOI [10.1021/jf9808426](https://doi.org/10.1021/jf9808426).
- Xia M, Li Y. 2021.** Complete chloroplast genome sequence of *Adenostemma lavenia* (Asteraceae) and phylogenetic analysis with related species. *Mitochondrial DNA Part B* **6**:2134–2136 DOI [10.1080/23802359.2021.1944369](https://doi.org/10.1080/23802359.2021.1944369).
- Xin T, Li X, Yao H, Lin Y, Ma X, Cheng R, Song J, Ni L, Fan C, Chen S. 2015.** Survey of commercial *Rhodiola* products revealed species diversity and potential safety issues. *Scientific Reports* **5**:8337 DOI [10.1038/srep08337](https://doi.org/10.1038/srep08337).
- Xiong C, Hu ZG, Tu Y, Liu HG, Wang P, Zhao MM, SH YH, Wu L, Sun W, Chen SL. 2016.** ITS2 barcoding DNA region combined with high resolution melting (HRM) analysis of Hyoscyami Semen, the mature seed of *Hyoscyamus niger*. *Chinese Journal of Natural Medicines* **14**:898–903 DOI [10.1016/s1875-5364\(17\)30014-6](https://doi.org/10.1016/s1875-5364(17)30014-6).
- Yang YL, Guo SJ, Ma RJ, Wang ZL. 2007.** Chemical composition of the volatile oil in *Adenostemma lavenia* (L.) O Ktze. *Journal of Tropical and Subtropical Botany* **15**:355–358.
- Yang BC, Lee MS, Sun FC, Chao HH, Chang WT, Lin MK, Chen HJ, Lee MS. 2020.** Rapid identification of the indigenous medicinal crop *Cinnamomum osmophloeum* from various adulterant *Cinnamomum* species by DNA polymorphism analysis. *Pharmacognosy Magazine* **16**:64–68 DOI [10.4103/pm.pm_267_19](https://doi.org/10.4103/pm.pm_267_19).
- Zhu S, Li Q, Chen S, Wang Y, Zhou L, Zeng C, Dong J. 2018.** Phylogenetic analysis of *Uncaria* species based on internal transcribed spacer (ITS) region and ITS2 secondary structure. *Pharmaceutical Biology* **56**:548–558 DOI [10.1080/13880209.2018.1499780](https://doi.org/10.1080/13880209.2018.1499780).