An effective protein extraction method for two-dimensional electrophoresis in the anticancer herb *Andrographis paniculata* Nees.

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Abstract

Proteomic analysis of plants relies on high yields of pure protein. In plants, protein extraction and purification present a great challenge due to accumulation of a large amount of interfering substances, including polysaccharides, polyphenols, and secondary metabolites. Therefore, it is necessary to modify the extraction protocols. A study was conducted to compare four protein extraction and precipitation methods for proteomic analysis. The results showed significant differences in protein content among the four methods. The chloroform–trichloroacetic acid–acetone method using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer provided the best results in terms of protein content, pellets, spot resolution, and intensity of unique spots detected. An overall of 83 qualitative or quantitative significant differential spots were found among the four methods. Based on the 2-DE gel map, the method is expected to benefit the development of high-level proteomic and biochemical studies of *Andrographis paniculata*, which may also be applied to other recalcitrant medicinal plant tissues.

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1. Introduction

The Kalmegh, *Andrographis paniculata*, is an important medicinal plant belonging to the family Acanthaceae [1, 2]. The production of diterpenes, flavonoids, and stigmasterols, such as andrographolide, neoandrographolide, and 14-deoxy-11, 12-didehydroandrographolide, represents the main active compounds used in the pharmaceutical industry [3,4]. The herb has exhibited a wide scope of pharmaceutical benefits, such as anti-HIV [5], anti-H1N1 [6], anticancer [7], and antihepatitis [8] properties.

The biodiversity of herbs using various marker systems has been subjected to very recent studies [9,10], but most likely the lack or reduced portion of protein-based markers in genetic investigations of this species could be related to the absence of an efficient extraction protocol for gaining high-quality pure proteins from *A. paniculata* tissues. Protein extraction and purification is the most critical step for proteomic analysis, especially from plant materials due to accumulation of a large amount of interfering substances such as polysaccharides, polyphenols, and secondary metabolites, which lead to many technical problems in protein purification and separation [11–13]. Many sample preparation protocols in 2-DE gel analysis including phenol–ammonium sulfate–methanol [12],

Abbreviations: *2-DE, two-dimensional electrophoresis; 2-ME, two-mercoptoethanol; APS, ammonium persulfate; BPB, bromophenol blue; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IGP, immobilized pH gradient; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TEMED, N,N,N,N-tetramethylenediamine.*

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phenol ammonium acetate [14, 15], chloroform [16, 17], and trichloroacetic acid (TCA)–acetone [18] have been reported, but they are not usable for all medicinal plants. Therefore, it is necessary to modify the extraction protocols. A perfect method should be highly reproducible and represent the highest number of protein types, with the lowest amount of contaminants, protein degradation, and modifications.

The present study was carried out to evaluate different extraction methods for a 2-D gel electrophoresis pattern in terms of protein yield, number of spots, image quality, and reproducibility. Thus, our main purpose was to improve the quality and purity of the extracted proteins from *A. paniculata* for 2-DE by modifying some parts of the procedures as well as the extraction buffer composition. For this reason, the TCA–acetone, phenol, phenol–ammonium sulfate–methanol, and combinations of TCA–acetone and chloroform protocols were compared with each other. To the best of our knowledge, this is the first published report on protein extraction from *A. paniculata*, and we expect that this modified protocol will be a significant stride toward future studies in proteome analysis of the species.

2. Materials and Methods

2.1. Plant material

A. paniculata leaves were used in all experiments. Fresh and fully expanded leaves (5 g) from 5 to 10 healthy 45-day-old plants of each accession were collected and thoroughly washed with deionized water, and were then frozen in liquid nitrogen and stored at −80 °C until extraction.

2.2. Protein extraction and solubilization methods

Two grams of the frozen leaf samples of each accession was ground into fine powder using the autoclaved and precooled mortar and pestle, and then homogenized with four different extraction buffers as follows: the phenol–ammonium acetate–methanol (method 1) described by Hurkman and Tanaka [14] (see Supporting Information Table A1), the TCA–acetone (method 2) described by Damerval et al. [18] (see Supporting Information Table A2), the phenol-ammonium sulfate-methanol (method 3) described by Saravanan and Rose [12] (see Supporting Information Table A3), and a modified system using chloroform–TCA–acetone based on 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (method 4).

2.3. Combination of chloroform–TCA–acetone using HEPES buffer

Two grams of the fine powdered leaf samples was homogenized with 10 mL of each extraction buffer [20 mM HEPES/KOH (Merck, Darmstadt, Germany), pH 7.5, 40 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, and 1 mM phenylmethanesulfonyl fluoride (PMSF)] (see Supporting Information Table A4). The samples were vortexed for 5 Min and centrifuged at 20,000*g* for 20 Min at 4 °C five times. The supernatants were collected in a sterile tube, ¼ volumes of chloroform were added and mixed, and kept for 5 Min at room temperature, and then centrifuged at 16,000*g* for 15 Min at 4 °C. The supernatants were collected in sterile tubes and precipitated with 4 volumes of 10% TCA containing 65 mM dithiothreitol (DTT) at −20 °C overnight. After centrifugation at 20,000*g* for 20 Min at 4 °C, the pellets were washed three times with ice-cold acetone containing 65 mM DTT. The pellets were air dried and solubilized in special solubilization buffer (containing 9.8 M Urea, 4% CHAPS, 65 mM DTT, and 200 mM Tris base) (see Supporting Information Table A5). The solubilized samples were dissolved in 500 μL rehydration buffer (see Supporting Information Table A6). The total protein contents were determined by the Bradford method [19] employing bovine serum albumin (Sigma-Aldrich, USA) as the standard. The measurements were made in triplicate at 595 nm, using a PerkinElmer (USA) Lambda25 UV/Vis spectrophotometer. Approximate protein sample quantification was necessary so that the same amount of total proteins could be separated across all gels.

2.4. Protein separation using SDS-PAGE

To establish extraction efficiencies, the protein samples were run on SDS-PAGE separation following the method of Laemmli [20]. Twenty micrograms of solubilized proteins was loaded in each lane of the 12% concentrated separating gel. Electrophoresis was accomplished at 100 V over 90 Min using a Bio-Rad (USA) Mini Protein electrophoresis system.

2.5. First and second dimension protein separation

The electrophoresis technique used in the current study was isoelectric focusing (IEF) and SDS-PAGE. The individual proteins in the mixture were separated based on their isoelectric point with a pH gradient in an electric field in the first dimension by IEF and based on molecular weight in the second dimension by SDS-PAGE. The pH gradients were generated with amphoteric molecules known as carrier ampholytes.

Protein mixtures were resolved using two-dimensional gel electrophoresis as described by O'Farrell [21]. Approximately 200 μg of total proteins extracted from the leaves of *A. paniculata* seedlings was solubilized in 20 μL solubilization buffer, incubated for 30 Min at room temperature, then mixed with 250 μL of rehydration buffer (9.8 M urea, 2% CHAPS, 0.5% IPG buffer, 65 mM DTT, and 0.1% bromophenol blue (BPB)), and 125 μL of the sample was then pipetted onto an IPG strip gel (pH 3–10; Bio-Rad), using the one channel rehydration tray in a bead-like manner. Each IPG strip was located on each channel gel side down and was rehydrated at room temperature for 14–16 H. After 16 H of rehydration, the strips were transferred to an IEF tray for the IEF. Two wet paper wicks were placed on the electrode in the IEF tray to remove salts and other interfering compounds from the proteins so that they do not interfere with the protein focusing. The first dimension (IEF) focusing was performed on the IEF Mini-protean II (Bio-Rad) with a program consisting of 250 V for 2 Min, 500 V for 30 Min, 1,000 V for 1 H, 4,000 V for 2 H, and a final focusing of 14,000 volt-hour focusing step at 4,000 V.

At the end of the IEF program, the IPG strips were first equilibrated with equilibration buffer for 15 Min [6 M urea, 50 mM Tris–HCl, pH 8.8, 2% SDS, 30% glycerol, and 2% DTT (w/v)] to reduce any disulfide bridges (see Supporting Information Table A7). Immediately after the first equilibration step, alkylation was carried out by incubating each strip in the second equilibration buffer with 2.5% of iodoacetamide to destroy all tertiary structures (see Supporting Information Table A8).

The second-dimensional electrophoresis was performed using the Laemmli method [20] on 12% polyacrylamide gels (Bio-Rad). The Mini-protein II gel system from Bio-Rad was used to separate proteins in the second dimension. After equilibration, the strips were placed in front of the plate and covered with 8% overlay agarose. Electrophoresis separation was carried out for approximately 90 Min at 100 V using the running buffer (3% Tris base, 14.4% glycine, 1% SDS).

2.6. Staining and digitization of protein pattern

Before staining, the gels were fixed overnight in a fixative solution (30% ethanol/10% acetic acid) on a shaker at 100 rpm (SASTEC Model: ST-344). The gels were then stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) in 40% (v/v) methanol and 7% (v/v) acetic acid for 1 H and destained with 40% (v/v) methanol and 7% (v/v) acetic acid until the background was clear. The gels were scanned and visualized using a densitometer (GS-800; Bio-Rad). Scanned gel images were exported to PDQuest software to evaluate the 2-DE gel and suitability of the protein extraction methods. The analysis was evaluated by visual inspection, focusing on those spots present in all three biological replicates for each protocol, showing qualitative or quantitative statistically significant differences between protocols. For each spot normalized mean, spot volume, SD, and coefficient of variation were determined.

2.7. Statistical analysis

SPSS software No.19 was employed to perform the statistical analysis. The data were analyzed by analysis of variance (ANOVA), and mean values from the four extraction protocols were compared by Duncan's multiple range test ($P \leq 0.01$).

3. Results

3.1. Comparison of different protein extraction methods

The four methods of sample extraction were compared in terms of protein yield, number of protein bands, spot focusing and resolution, number of spots resolved, intensity, and variability. Leaf extracts obtained using the four methods were compared for protein contents using the Bradford assay. The results showed that the weight of the final pellet obtained from 1 g of the fresh leaf tissue using different methods was statistically significant (Table 1). ANOVA also showed that there were significant differences among the various protein extraction methods in terms of total protein ($P \leq 0.01$). A higher protein yield was obtained with method 4 (1.144 ± 0.045 mg/g fresh weight), whereas the lowest was obtained with method 3 (0.175 ± 0.020 mg/g fresh weight) (Table 1). The SDS-PAGE analysis showed that the protein patterns among the four different extraction methods were different, and the quantity and quality of proteins in samples from method 4 were higher compared with other methods. Protein analysis showed that 11 different types of proteins were detectable in *A. paniculata* leaves (Fig. 1).

From the results of initial experiments using IPG strips with different pH ranges, it was observed that most of the spots were concentrated in the pH region of 4–7. Thus, it was concluded that the IPG strips with pH ranging from 4 to 7

TABLE 1 ***Comparison of different protein extraction methods based on protein content, pellets, and number of spots***

Method	*Protein content (mg/g fresh leaf ± SEM)*	*Pellet (mg/g fresh leaf ± SEM)*	*Number of spots*
1	0.240 ± 0.015^{a}	7.72 ± 0.42^{a}	51 ± 2.89^{a}
2	0.408 ± 0.020^{b}	12.43 ± 0.50^{b}	70 ± 4.04^{b}
3	0.175 ± 0.020^{a}	5.90 ± 0.51^{a}	55 ± 3.46^{a}
4	1.144 ± 0.045^{c}	33.04 ± 1.27^{c}	83 ± 4.62^{c}

The data are mean values of three independent measurements and SEM. Values superscripted by different letters are significantly different by Duncan's multiple range test ($p \leq 0.01$).

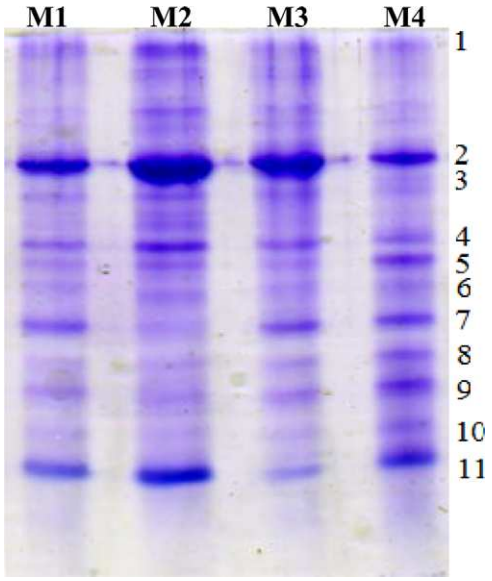

FIG. 1 *SDS-PAGE polypeptide profile of the leaf protein from A. paniculata using four different protein extraction methods. M1 represents method 1, M2; method 2, M3; method 3, M4; method 4; numbers 1–11 represent the different protein bands.*

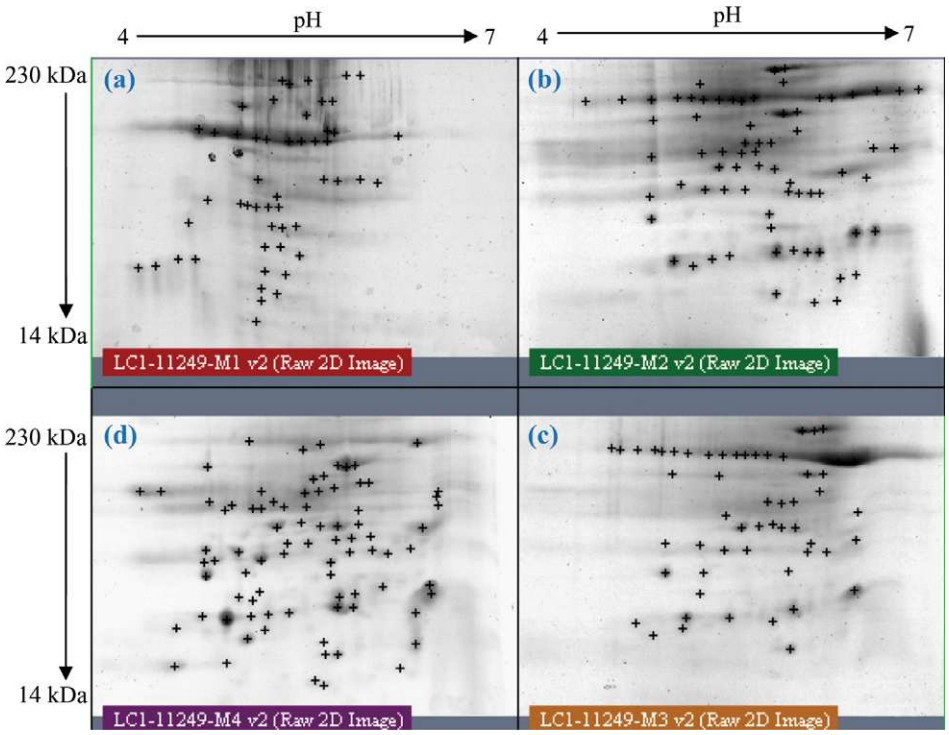

FIG. 2 *Real gels of protein extracts obtained using different protein extraction methods. The phenol (a), the TCA–acetone (b), the phenol-ammonium sulfate-methanol method (c), and a combination of TCA–acetone–chloroform using HEPES (d) are presented. A 100 μL protein sample (100 μg) was separated over pH range 4–7 (7 cm strips) and 12% SDS-PAGE. Numbered spots correspond to those showing qualitative or quantitative significance.*

produced higher resolution of proteins in comparison with the pH range of 3–10. Subsequently, in all main samples IPG strips with pH ranging from 4 to 7 were used. In each method, a real gel was evaluated together with a master gel (Figs. 2a–2d). Higher resolution, minimal horizontal streaking, and much better focusing of the spots were observed with method 4 compared with other extraction methods. The total number of resolvable spots by 2-DE varied with different methods. Proteins extracted by method 4 had the highest resolution with substantially reduced horizontal streaking of 2-DE gels compared with other methods (Fig. 2d). The average number of detected spots among the three replicates for each method was: 51 $\pm$ 2.89 (method 1), 70 $\pm$ 4.04 (method 2), 55 $\pm$ 3.46 (method 3), and 83 $\pm$ 4.62 (method 4) (Table 1), indicating a higher reproducibility with method 4. As mentioned earlier, no single method of extraction is suitable for all types of different plant materials. This can be attributed to the differences in sample complexity and in the dynamic range of protein abundance in samples. Nevertheless, there was a high correlation between the large number of detected spots and protein content, which has been previously reported [22].

The total number of spots showed qualitative or quantitative differences between the different methods. Some spots were absent (qualitative differences) or overrepresented in a specific method (quantitative differences) (Figs. 2a–2d). Methods 1, 2, and 3 not only allowed the visualization of lower intense spots (qualitative differences), but also decreased the intensity of common spots (quantitative differences). However, compared with methods 1, 2, and 3, method 4 was more complex and time consuming, considering its irrefutable efficiencies such as improving protein solubilization, handling a greater number of samples, producing a higher number of protein spots on 2-D gel, removing contaminants, and providing high-quality protein samples from *A. paniculata* leaves, it still can be regarded as an appropriate method for similar proteomics studies in the future. Thus, the alternative buffer-based protocols are very valuable for proteome descriptive objectives (Table 1 and Figs. 2a–2d).

3.2. Analysis of protein spots using the PDQuest software

To compare the proteins from the different gels, gel matching was carried out using the image analysis software, PDQuest 8.0.1 (Bio-Rad). The PDQuest software allowed the detection of protein spots, with background subtraction and the matching of the spots between samples from the different methods. The first step in using PDQuest was to filter and transform the gels to remove the extra background of the gel. All the detection parameters were determined by selecting a small, faint spot and the largest spot on the master gel. During this stage, additional filtering by horizontal and vertical streaking was also performed. At least three gels replicated from each

method were aligned to create a composite image containing only those proteins that are common to all three gels.

The component gels created a complex image that contained the spots commonly present on all gels. The images from the different methods were aligned to create a supercomposite image using the matching tools. A typical supercomposite image of the leaf samples is shown in Figs. 2a–2d. Analysis of the detected spots using PDQuest software revealed different numbers of proteins in the different methods.

4. Discussion

A. paniculata Nees. is an important medicinal plant. Therefore, it is necessary to preserve the genetic resources of *A. paniculata*, integrating the conservation issue with sustainable exploitation. Nonetheless, this can be achieved only once the existing genetic diversity of the plant becomes evident. It should be recalled again that the herb's biodiversity issue has been assessed by different markers, but unfortunately, protein markers have been neglected in this area. Protein markers have been employed to evaluate the genetic variability in plants, and the extraction methods have been a necessary prerequisite in this trend. Thus, these approaches require protocols that can efficiently extract proteins of both high quality and quantity. This is a great challenge in *A. paniculata*.

In the present study, three standard methods and an improved method (method 4) were evaluated for protein extraction from *A. paniculata* leaves by two-dimensional electrophoresis. The SDS-PAGE results demonstrated low background interference of gels using method 4 because of the absence of nonprotein contaminants, suggesting that high-quality protein samples were obtained with this method. In the first and third tested methods, no high-quality protein was extracted (Table 1), which proved to be inadequate. This is probably because of the presence of polyphenols, polysaccharides, and other secondary metabolites. In addition, protein losses in these methods (1 and 3) during sequential washes (with ammonium sulfate or ammonium acetate in methanol and acetone) could be due to washing of pellets in the absence of DTT or 2-ME, which denatures the proteins by reducing disulfide linkages.

In the second method, the addition of TCA–acetone allowed an increase in protein precipitation and the protein content (Table 1). Although the protein content was increased with method 2, the obtained pellets using the TCA–acetone method were more difficult to dissolve in solubilization buffer due to the coextraction of polymeric contaminants [23], as these contaminants precipitate with protein and cannot be removed by the final washing steps and some materials were always insoluble. Similar results were reported by Islam et al. [24], who showed poorly detected spots in 2-DE for rice leaves extracted with TCA–acetone. Thus, a further optimization was obtained by subjecting the supernatants to chloroform (method 4), and the addition of chloroform allowed an increase in the precipitation of nonprotein components and secondary metabolites (such as polyphenols, lipids, and pigments), resulting in a change in the color of the supernatant from light brown to white.

Also, the addition of TCA–acetone, including DTT, as performed in method 4, increased protein precipitation and protein content by 1.144 mg/g fresh leaf in comparison with other methods (Table 1). In this method, centrifuging the samples at 20,000*g* for five times led to the removal of the cell walls and vacuole materials. The obtained results showed that the use of the organic solvent (chloroform) had substantially removed nonprotein components and secondary metabolites, thus facilitating the process of extraction, which led to better protein quality and higher protein content. To avoid protein precipitation during the extraction process and improve protein solubilization, the pH of the extraction buffer was adjusted to 7.5. As observed with method 2, the obtained pellets using the TCA–acetone method were more difficult to dissolve in the solubilization buffer. Hence, to resolve this problem, the protein pellets were first washed with distilled water containing DTT and were then washed again with ice-cold acetone. This step proved to be very critical for the recovery of pure proteins in the entire isolation process. Additionally, the protein pellets were solubilized in a modified solubilization buffer containing 9.8 M urea, 4% CHAPS, 65 mM DTT, and 200 mM Tris base. Thus, the combination of chloroform and TCA–acetone using HEPES buffer with three washings with ice-cold acetone containing DTT (method 4) resulted in higher purity of the extracted proteins. The method proved to be very effective for extracting sufficient quantities of high-quality proteins from *A. paniculata* leaves.

The results suggested that using TCA–acetone alone in the extraction process was not sufficient to remove nonprotein components and improve the quantity and quality of proteins. Thus, the combination of PMSF as a protease inhibitor, and glycerol as a stabilization agent of the protein in the extraction buffer along with washing of the supernatant with chloroform and precipitation of protein with TCA–acetone and washing with distilled water and acetone containing DTT as a reducing agent, to overcome some form of tertiary protein folding and break up quaternary protein structure proved to be very effective to extract sufficient quantities of high-quality protein from *A. paniculata* leaves.

A possible explanation for the differential spot patterns detected might be due to the different effects of the physical or chemical environment of specific proteins, changing protein stability or solubility differences in the extraction methods. The extraction method using chloroform–TCA–acetone led to selective solubilization and extraction of specific proteins. These results are in agreement with that of Saravanan and Rose [12], who reported that extracted protein using TCA–acetone usually contained fewer proteins with higher molecular weight, whereas chloroform–TCA–acetone extraction generated samples with resolved bands over a wider range of molecular weight. The use of organic solvents like chloroform and phenol have been previously reported to be suitable for the removal of secondary metabolites like polyphenol compounds from

protein solutions by increasing their solubility, and thus preventing their coprecipitation with protein [25–27].

5. Conclusion

To establish a routine procedure for the application of proteomic analysis, considering the characteristics of *A. paniculata*, an effective protein extraction method using chloroform–TCA–acetone with HEPES buffer was developed. The method efficiently improved protein solubilization and yielded a greater number of protein spots on 2-D gel. The improved method effectively removed contaminants and provided high-quality protein samples from the *A. paniculata* leaf. The gel electrophoresis images were well resolved, with less streaking, and a greater number of spots noted on 2-DE gels. Although the improved method was more complex and consequently more time consuming, success in gaining high-quality protein samples outweighed the time spent on sample preparation. Therefore, it is expected that the method described here will be useful in the development of high-level proteomic and biochemical studies in *A. paniculata* as well as in other recalcitrant medicinal plant tissues.

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7. References

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