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A Simple and Facile Method for the Isolation of Andrographolide from *Andrographis Paniculata* Nees

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ABSTRACT

Andrographis paniculata is an important medicinal plant in Indian and Chinese systems of medicine used for a number of ailments. Andrographolide (AP), a diterpene lactone, has been reported to be responsible for many of the activities. A number of methods are available for the isolation of AP. However, they are time consuming and not cost effective. We employed five relatively simpler methods for the isolation of AP. The identities and purity of the compounds isolated by these five methods were established by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), melting point, ultraviolet (UV), infrared (FTIR), mass, and nuclear magnetic resonance (^{1}H NMR) spectra. Treatment of the extracts with activated charcoal proved to be quite effective in removing all coloring matter and resulted in isolation of pure AP. Of the five methods, prior maceration with methanol for 16 h followed by refluxing for 1h yielded 0.6%w/w of AP and was found to be quite satisfactory. This method does not need prior extraction of the powder with toluene nor does it require washing of the concentrated extract with toluene. In this method only methanol (MeOH) was employed. All the spectral data and melting point of andrographolide isolated by this method confirmed its purity. This method is simple as it involves only three steps and has the potential for commercial scale up.

Keywords: *Andrographis paniculata* Nees, Andrographolide, FTIR, Mass, ^{1}H NMR, HPLC.

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INTRODUCTION

Andrographis paniculata Nees (Family: Acanthaceae) commonly known as *Kalmegh,* is an annual herb growing throughout India and China. It has been reported to be effective in liver disorders, inflammation, infections, dysentery, common cold, diabetes, cancer etc[1]. It contains mainly a diterpene lactone andrographolide (AP) along with some other compounds such as 14-deoxy-11, 12-dehydroandrographolide, 14-deoxyandrographolide, kalameghin etc[2, 3]. Andrographolide (Figure 1) is the major compound to which most of the pharmacological activities of the herb are attributed. A number of methods have been reported for the isolation of AP. Mohan et al.[4] employed a microwave assisted method using water and MeOH as solvents. Rajni et al.[5] employed cold maceration with a mixture of dichloromethane (DCM): MeOH (1:1). The extract was concentrated to a syrupy mass and repeatedly washed with toluene to remove green color. Repeated crystallization was done in MeOH to obtain pure AP. Borooah and Borua[6] used MeOH extract of *A. paniculata* which was chromatographed on silica gel column and eluted with a gradient of chloroform ($CHCl_3$) and MeOH. Majee et al.[7] fractionated MeOH extract of A. *paniculata* leaves in ethyl acetate (EtOAc) and water. EtOAc extract was partitioned with n-butanol which gave a precipitate of crude AP; it was further purified by column chromatography on silica gel using $CHCl_3$ and MeOH. Chao and Lin[8] extracted the whole plant powder of *A. paniculata* in 95% ethanol, fractionated into EtOAc soluble fraction by liquid –liquid extraction and from it, AP was isolated by silica gel column chromatography. Kumoro and Hasan[9] reported soxhlet extraction of the herb with MeOH or extraction with super critical carbon dioxide to be the best choice for isolation of pure AP. There are reports where whole plant material of *A. paniculata* was extracted by refluxing with MeOH and quantity of AP in the extract was determined by HPLC (Sharma et al.[10]; Katta et al.[11]). Song et al.[12] extracted the herb with 70% ethanol coupled with sonication for HPLC quantification of AP. But the above methods involve a number of steps and are time consuming. In the present study, we made an attempt to isolate andrographolide in a crystalline form by simpler methods which may be commercially feasible for scale up.

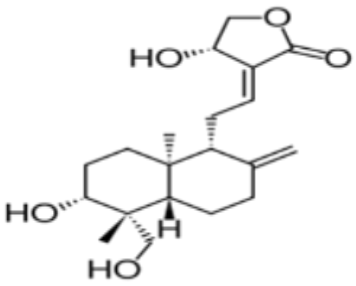

Figure 1: Andrographolide

MATERIALS AND METHOD

Instruments

Melting points were uncorrected and taken in an open capillary on a Toshniwal (Mumbai, India) melting point apparatus.

TLC was performed on aluminium plates precoated with silica gel $60F_{254}$ (cat. No.1.05548, E.Merck, Darmastadt, Germany). A Digital analytical balance (Wenstar DA13-220) was used for weighing andrographolide. Samples were applied on plates and developed in glass chamber (20X10X4 cm). UV spectra of the standard and all the isolated compounds were taken in MeOH on a Shimadzu UV-visible spectrophotometer, model 1800 with UV-Probe software version 2.31. IR spectra were recorded on a Shimadzu FTIR-8400S with ATR-MIRACLE 10 spectrophotometer. Mass spectra (MS) were taken on an Advion Expression CMS (Compact Mass Spectometer) using ESI as ionization source. ^{1}H NMR spectra were recorded on a 400 MHz Bruker Protonon NMR Spectrometer. HPLC was performed on an isocratic Shimadzu LC2 2016 HPLC instrument by using C18 elegant column (2.5 mm diameter: 5 µm particle size and 25 cm length) with a quadruple pump and UV detector.

Plant material

Dried herb consisting of stem and leaves of *A. paniculata* was procured from Yucca enterprise, Mumbai, India. The identity of the plant was established by comparing it with the plants growing in the experimental garden of the author's institute and by comparing the microscopic characters with those reported in the literature[13, 14]. A specimen of the herb was preserved in the Pharmacognosy museum of the author's institute (Specimen No. LJIP/ cognosy/AP 01/2014). The entire dry herb was reduced to a powder of #40 and stored in an air tight amber colored glass container.

Reagents

All the chemicals used in the isolation were of laboratory grade and obtained from S.D. Fine Chemicals, Mumbai, India or from Qualigens, Mumbai, India. Chemicals used for TLC, UV, NMR and HPLC were of analytical grade.

Procurement of standard Andrographolide (AP)

Reference standard of andrographolide (AP; 98% pure) was gifted by Avance phytotherapies Pvt. Ltd., Ahmedabad, India.

Methods for Isolation of Andrographolide

Five different methods, including the method of Rajani et al.[5] with some modification were

employed for the isolation of AP. All isolations were done in triplicates. Per cent yields; melting points, TLC and HPLC chromatograms, UV, FTIR, Mass and ^{1}HNMR spectra were taken for the isolated compounds.

Method 1

50 g of *A. paniculata* powder was initially extracted in cold with 200 mL of toluene for 24 h. The solution was filtered under vacuum. The marc was dried at room temperature to remove toluene. It was extracted by refluxing in MeOH three times. Refluxing was done for 1 h each time employing 200 mL of MeOH. The extracts were pooled, stirred with 1 g activated animal charcoal and filtered through Whatman No. 1 filter paper. MeOH was evaporated at 70º C on a water bath. The resulting solid crystalline mass was washed with 10-15 mL of cold MeOH and filtered. The crude crystals were again washed with 2 x 10 mL cold MeOH which yielded white crystals (AP1).

Method 2

50 g of *A. paniculata* powder was initially defatted and decolorized with toluene as described in method l. The marc was refluxed thrice for 1 h each time with 200 mL of a mixture of DCM : MeOH (1: 1) and filtered. The extracts were pooled, treated with 1 g activated animal charcoal and filtered through Whatman No. 1 filter paper. The filtrate was evaporated at 70º C on a water bath which yielded a solid crystalline mass. Pure white crystals (AP 2) were obtained after washing the crystalline mass with 2 x 10 mL of cold MeOH.

Method 3

50 g of *A. paniculata* powder was first extracted with toluene as above and the dried marc was refluxed thrice for 1 h each time with 200 mL EtOAc and filtered. Activated animal charcoal was added to the combined extracts, stirred for 5 minutes and filtered through Whatman No. 1 filter paper. The residue, on evaporation of EtOAc, was washed with 2 x 10 mL cold MeOH to get crystals (AP 3).

Method 4

50 g of *A. paniculata* powder was macerated in 200 mL MeOH for 16 h and then refluxed for 1 h. The extract was filtered. The extract was treated with activated animal charcoal and filtered through Whatman No. 1 filter paper. MeOH was evaporated at 70º C on a water bath. The resulting solid crystalline mass was washed with 2 x 10 mL of cold MeOH and filtered to get white crystals (AP4).

Method 5

The method as reported by Rajani et al[5] was followed with some modification. 50 g *A. paniculata* powder was macerated in 200 mL mixture of DCM: MeOH (1:1) for 24 h and then refluxed for 1 h

and filtered. The marc was further refluxed twice with 200 mL of the same solvent for l h each time. Instead of evaporating the extract and washing the green gummy residue with toluene several times as reported in the method, we treated the extract with activated animal charcoal and filtered. Complete evaporation of the solvents yielded crude crystals. These crystals were washed with 2 x 10 mL of cold MeOH and filtered. White needles of AP (AP 5) separated out.

Confirmation of identity and purity of isolated andrographolides

UV spectra

10 mg each of the standard (AP), AP1, AP2, AP3, AP4, and AP5 were dissolved in 5 mL MeOH and made to 10 mL with MeOH to obtain a standard stock solution (1000 µg/mL).

Further dilution of this stock solution was done with MeOH to get a concentration of 20µg/mL. These solutions were scanned between 200-400 nm. The spectra were recorded and superimposed.

Thin layer chromatography (TLC)

TLC of isolated compounds was performed along with standard AP on a silica gel plate using $CHCl_3$: MeOH: EtOAc (8:1.5:1) as mobile phase. 20 µl of the above stock solutions were spotted. The plate was run in MeOH and dried in an oven at 100°C for 5 minutes before application of the sample. The spots were visualized under UV light at a wavelength of 254 nm.

High performance liquid chromatography (HPLC)

Sample solutions were prepared as described earlier. The solutions were filtered. The HPLC chromatograms of standard and isolated compounds were taken in a mobile phase containing MeOH: water (65:35); flow rate 1mL/min and detection at 225nm.

Fourier transfer infra-red spectroscopy (FTIR)

Each individual sample was directly placed on to the ATR crystal surface of the instrument and IR spectra were taken in the range of 400-4000 cm^{-1}. Data was analyzed by using IR solution version 1.20.

Mass spectra

Mass spectra were taken on an Advion Expression CMS (Compact Mass Spectometer) using ESI as ionization source

Nuclear magnetic resonance spectra (NMR)

Proton NMR spectra of the isolated compounds were recorded on a 400 MHz Bruker NMR spectrometer in $CDCl_3$.

RESULTS AND DISCUSSION

The different methods yielded 0.5 -0.6% w/w of pure andrographolide (Table 1). The melting

points of all the isolated compounds ranged between 225 -230°C which are within the range reported in the literature[15]. The standard (AP) and isolated compounds exhibited λ_{max} at 225 - 226 nm. The overlain UV spectra are shown in figure 2.

Table 1: Extractive Values, Yield (%w/w) and Melting Points of Andrographolide Isolated by Different Methods

Method	% (w/w)Extractive Value	Yield *(% w/w) and Product Code	Melting Point
1	Toluene: 0.1; MeOH: 2.7	0.58± 0.025 (AP1)	225-230º C
2	Toluene:0.15 DCM: MeOH: 3.7	0.60± 0.023 (AP2)	230º C
3	Toluene: 0.2 EtOAc : 2.0	0.45± 0.028 (AP3)	225-230º C
4	**2.9**	**0.60± 0.025 (AP4)**	**230º C**
5	3.7	0.60± 0.026 (AP5)	225-230º C

*Mean ± S.E.M. of three experiments.

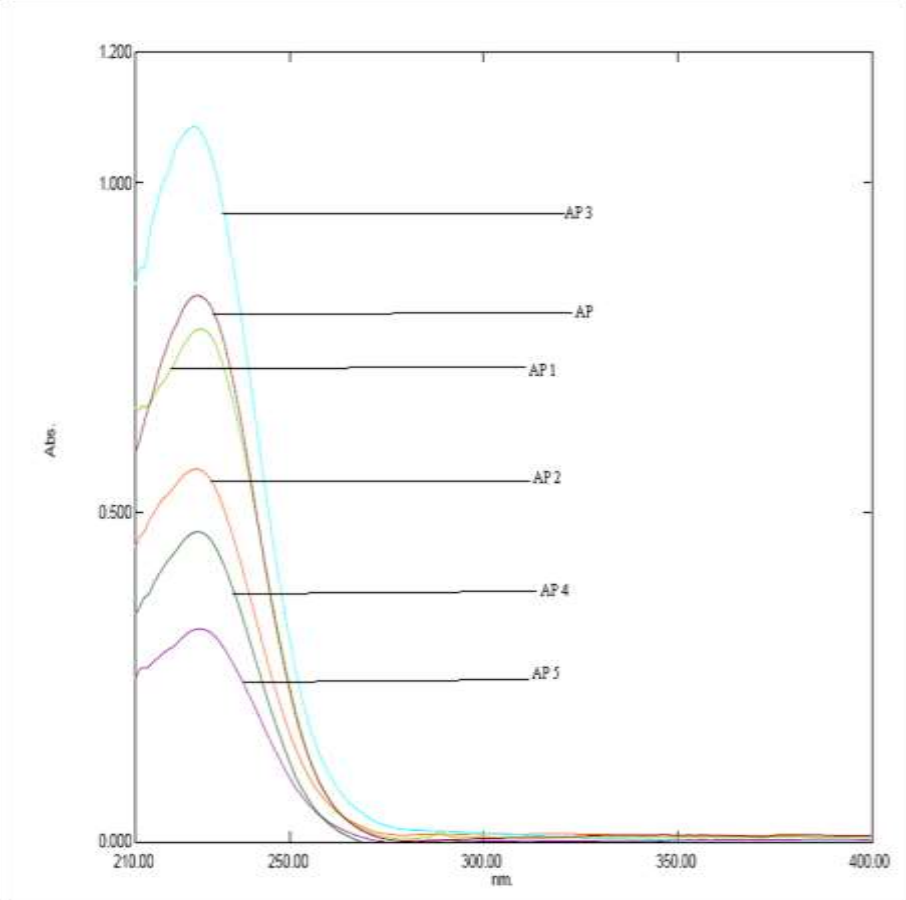

Figure 2: Overlay of UV spectra of AP isolated by different methods

All the isolated compounds and standard (AP) showed a single spot on the TLC with R_f 0.6 in the mobile phase. The HPLC analysis of isolated compounds gave a single peak with a retention time R_t 5.5minute. The HPLC chromatograms of the isolated and standard andrographolide are shown in figure 3.

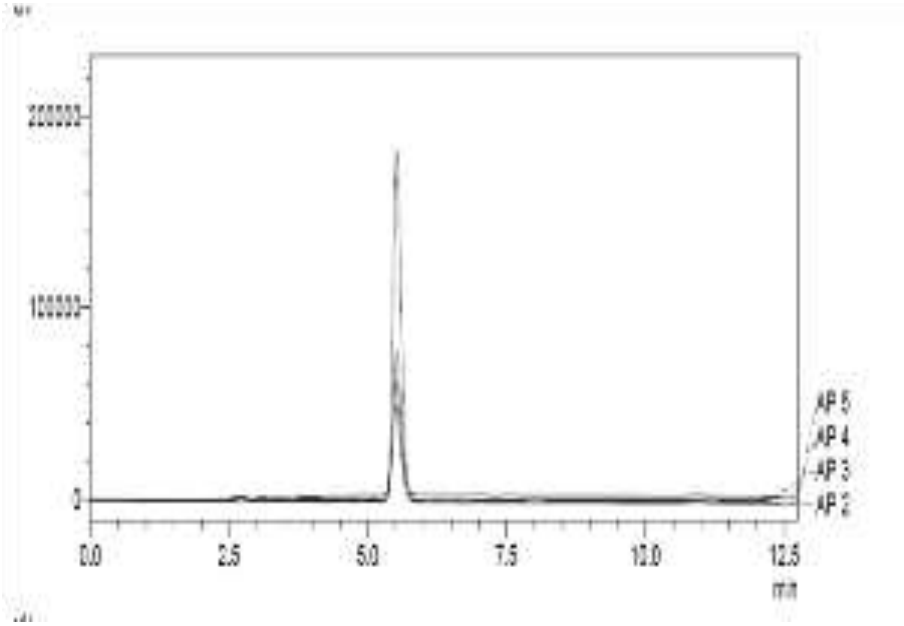

Figure 3A: Overlay HPLC chromatogram of AP isolated by different methods

FTIR spectra of andrographlide isolated by the five methods were similar. FTIR exhibited peaks at 655 cm $^{-1}$(C-C=C), 709 cm $^{-1}$ (C-C-C), 810 cm $^{-1}$ (C-C), 871 cm $^{-1}$(C-C-C), 902 cm $^{-1}$ (external CH_2), 979 & 1026 cm $^{-1}$ (O-H deformation), 1219 cm $^{-1}$ (C-O-C streching), 1674 cm $^{-1}$ (C=O stretching due to α,β-unsaturation), 1720 cm $^{-1}$ (lactone ring), 2846 & 2924 cm $^{-1}$ (C-H stretching), 3309 cm $^{-}$1(OH). This data is in complete agreement with the data available in the literature [16]. The mass spectra of the isolated compounds were in complete agreement with the spectra of standard andrographolide and those reported in the literature[12]. Major fragmentation peaks appeared at m/z 351, 333,315, 297, 285,257 in the compounds isolated by different methods. Proton NMR spectra showed shifts at δ 0.6 and 1.1-1.2 (each 3 H, S, 2 CH3), δ 3.2 (1 H, t, 19-H), δ 4.7-4.8 (each 1H, S, 17-H), δ 4.1-4.4 (each 1 H, dd, 15-H), δ 3.4 (1 H, m, 3β-H), δ 6.8 (1 H, t, 12-H) [16]. From the various physical, chromatographic and spectral data obtained in our studies, it is clear that the isolated andrographolides are pure. MeOH gave better extraction than EtOAc. Mixture of DCM and MeOH also yielded good extractive values. However, initial extraction of the powder with toluene was found to be more ideal than initial extraction with DCM: MeOH followed by number of washings with toluene as reported by Rajani et al[5]. Further, employing a single and cheaper solvent is ideal for scale up and commercial isolation of the compounds. Extraction with toluene effectively removed most of the waxy and coloring material. In our method (Method 4), extraction or washing with toluene was eliminated. Moreover, the material was macerated and later refluxed with MeOH only once for 1h. The extract was treated with activated animal charcoal and evaporated. Thus this method reduced number of solvents to be used

(only MeOH was used in place of MeOH, DCM, toluene used by Rajani et al.[5]) and the number of unit operations. None of the reported methods employed activated animal charcoal treatment for removing the color and other impurities. Activated animal charcoal treatment of the crude extracts resulted in colorless crystals and eliminated the need for several repeated washings and crystallization in hot MeOH as reported in the literature[5]. Thus this method (method 4) is simple, economical and has the potential for commercial isolation of AP.

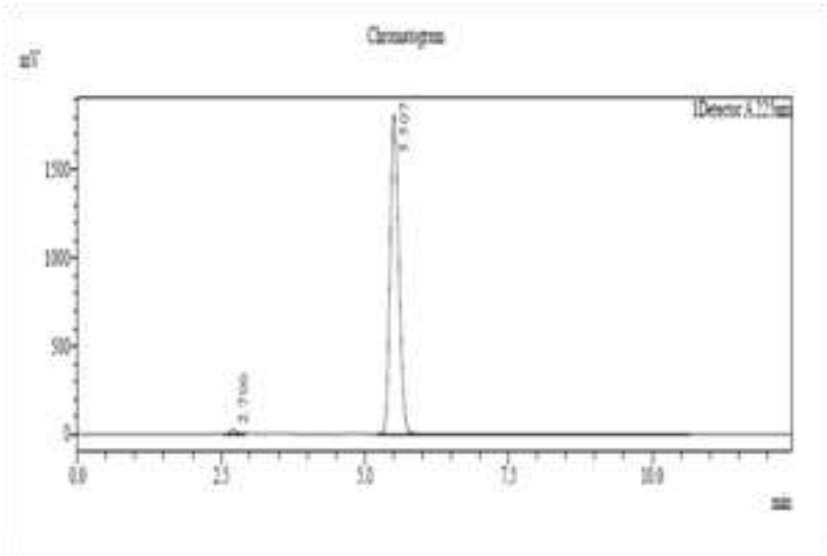

Figure 3B: HPLC chromatogram of Standard AP

CONCLUSION

Different methods of extraction of *A. paniculata* and treatment of the extracts were studied. A 16 h maceration with MeOH followed by 1 h refluxing only once and treatment with activated animal charcoal resulted in satisfactory isolation of pure AP. The method is compared with other methods including the ones reported in the literature. The compounds isolated by this and other methods were studied for their purity and homogeneity by noting melting points, UV, TLC, HPLC, FTIR, Mass and ^{1}H NMR spectra. Method 4 is simple and economical. It can be scaled up for commercial isolation of AP.

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