Comparison of green extraction methods with conventional extraction method for extract yield, L-DOPA concentration and antioxidant activity of *Mucuna pruriens* seed

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**ABSTRACT**

*Mucuna pruriens* is a plant of *Fabaceae* family. Seed of *M. pruriens* is considered as a rich source of levo-3,4 dihydroxyphenylalanine (L-DOPA), a non-protein phenolic amino acid. In the present study, three different extraction methods were compared for extract yield, concentration of bioactive compounds such as total phenol, L-DOPA and antioxidant capacity. Extracts were prepared using water acidified with hydrochloric acid (0.1 N) by conventional method of refluxing as well as two green methods namely ultrasound and microwave assisted solvent extraction. A rapid and qualified high-performance liquid chromatography method was also developed for quantification of L-DOPA in different extracts. Among the three extraction methods, microwave assisted extraction provided the best results for yield and quality of *M. pruriens* extract in much shorter time in comparison to refluxing method of extraction.

**Introduction**

*Mucuna* spp. has been described to contain many bioactive substances. About 15 species of *Mucuna* are found in the forests and plains of India (1). *Mucuna pruriens* (L.) belongs to the family *Fabaceae* and used for various purposes in traditional medicine in several countries (2, 3). The plant is an herbaceous twining annual, found in bushes and hedges at damp places, ravines and scrap jungles throughout the plains of India (4). Although all parts of the *Mucuna* plants have been reported to possess medicinal properties, great importance has been attached to seeds. Seed contains appreciable concentration of bioactive compounds such as free phenolics, tannins, phytic acid in addition to high protein content (26–29%) and other nutrients (5). In India, its mature seed is traditionally consumed by ethnic groups of Tamilnadu, Kerala, Karnataka and Andhra Pradesh (6). Seeds are used as a tonic and for male vitality in traditional medicine (7). Along with, *Pueria tuberosa, Orchis latifolia* and *Asparagus racemosus*, seeds of *M. pruriens* are used as an aphrodisiac. Also, in *Unani* and *Ayurvedic* systems of Indian Medicine, *M. pruriens* is used for the treatment of male impotency, in sexual debility and as nerve tonic (7, 8). Different preparations from seed are also used for the management of ageing, rheumatoid arthritis, diabetes, male infertility and nervous disorders (1, 9–11).

Seed of *M. pruriens* is considered as a rich source of levo-3,4 dihydroxyphenylalanine (L-DOPA, Figure 1), a nonprotein phenolic amino acid and precursor of the brain neurotransmitter dopamine (12–14). Seed extract have antiparkinsonian effect. Even, L-DOPA free fraction of *M. pruriens* seed showed significant antiparkinsonism activity (15, 16). L-DOPA has also been investigated as a dietary supplement to manage hypertension, renal failure, and liver cirrhosis (6). Protective effects of L-DOPA on small bowel injury, ulcer, gastrointestinal diseases, diabetes, etc. were also studied (5). Beside L-DOPA, nicotine, physostigmine, serotonin, bufotenine, choline, *N*-dimethyl tryptamine and some indole compounds are the other phytochemicals present in other parts such as roots, stems, leaves of *M. pruriens* (14, 17). In addition to L-DOPA, pharmacologically active compounds methylated and non-methylated tetra hydroisoquinoline are also present in *Mucuna* spp (18, 19). L-DOPA, if ingested in large amounts, is potentially toxic (20).

Standardization of herbal formulations is getting importance because of the indiscriminate use of herbal...
medicines. Extraction is an important step involved in the recovery of bioactive components from plant materials. To accelerate the extraction of bioactive compounds from plants, green extraction methods such as ultrasound assisted solvent extraction (UASE) and microwave assisted solvent extraction (MASE) are currently being used along with the conventional extraction techniques like maceration and Soxhlet extraction (21). Variation in the biological activities of extract obtained from different extraction techniques have been reported therefore selection of the suitable extraction method is important for obtaining the extracts with required pharmacological activities (22). There is a heavy demand of Mucuna in Indian drug market. Optimization of L-DOPA extraction and its quantification is important as ingestion of excessive amounts can lead to severe psychosis, nausea, emesis, arrhythmia, hypertension and dyskinesias (20, 23, 24).

Although, extensive research publications are available in the literature regarding isolation, characterization and pharmacological activities of L-DOPA from Mucuna species, a thorough literature search revealed lack of studies aimed for the extraction optimization of L-DOPA using both conventional and nonconventional extraction techniques. Keeping this in view, present investigation was undertaken with the following objectives (i) to study the effect of extraction methods on extract yield, total phenol content (TPC) and L-DOPA content in seed extracts of M. pruriens (ii) to develop a high-performance liquid chromatography (HPLC) method qualified in terms of limit of detection (LOD), limit of quantification (LOQ), accuracy and precision for providing qualitative as well as quantitative description of the extraction method and (iii) to apply developed and qualified HPLC method for quantification of L-DOPA in different extracts. Acidified water was used for the extract preparation since under the acidic conditions, softening of wall of tissue lead to release of bounded phenolic compounds. Along with the two green extraction methods such as UASE and MASE, conventional extraction method of refluxing was also used for comparison of the results. Further, to assess, the quality of the extracts prepared using refluxing, UASE and MASE methods yield, total phenolics, L-DOPA concentration and antioxidant capacities of extracts were compared.

Results and discussion

Selection of extraction method is very important for extract yield maximization with minimum loss of bioactive compounds and minimum consumption of organic solvents as well as energy. The mass of the obtained extracted materials were converted to a percentage based on how much extract mass would be obtained from 100 g of seeds to get the extract yield. It varied for different extraction methods used. Extraction using refluxing had higher extract yield in comparison to UASE and MASE possibly due to the exhaustive extraction (Table 1, Figure 2). Further, in case of UASE and MASE, in general, extract yield increased with increase of extraction time, however, no definite trend was obtained.

TPC in extracts prepared using three extraction method was calculated from the calibration curve made with gallic acid as reference. Calibration curve was represented by the linear equation $Y = 0.006X - 0.004$, $r^2 = 0.99$. TPC of the extracts was divided by the extract yield to calculate their percentage in the seed powder of M. pruriens (Table 1). TPC obtained in the seed samples were in close agreement with earlier reported values (3, 25, 26). TPC was maximum in extracts prepared by MASE and it was followed by extracts obtained from refluxing and UASE methods. In case of UASE extracts, TPC also increased with increase in extraction time. Similar type of observation was also reported earlier. It was observed for microwave treated two accessions of M. pruriens that increase in total free phenolics was time dependent (27). L-DOPA concentration in the extracts was determined using a modified and validated HPLC method. The validation parameters of the developed HPLC method are described in Tables 2 and 3. HPLC chromatogram of standard L-DOPA and extracts are shown in Figure 2. Mean retention time of L-DOPA was 4.06 ± 0.04 min. using the developed HPLC method, LOD and LOQ were found to be 15 and 25 μg/mL, respectively. Also the calibration curve prepared with standard L-DOPA was linear in large concentration range (25–500 μg/mL, Figure 3) with $r^2$ value greater than 0.99. HPLC chromatograms of the standard L-DOPA and extracts were shown in Figure 3. L-DOPA concentration in the different extracts was quantified on the basis of peak area of the HPLC chromatograms (Figure 2 (B-2E)). L-DOPA in the extract prepared using UASE and MASE had higher concentration than extract prepared using refluxing method. Antioxidant activity of the plant extract depends on the concentration of the antioxidant compounds as well as on the form of preparation. The antioxidant activity of the extract samples were

Figure 1. Structure of L-DOPA.
determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Results were expressed in terms of IC50 values of the extracts. As expected, *M. pruriens* seed extracts had high antioxidant capacity due to the high concentration of phenolics (Table 1). In the present study, IC50 values were lower than the values reported by Longhi et al. (3) for *M. pruriens* seed extract prepared using water acidified with citric acid keeping pH of the medium between 3.0 and 4.0, thereby, exhibiting better antioxidant capacity. MASE extracts of the *M. pruriens* seed had lowest IC50 values, thereby, highest antioxidant capacities (Figure 4).

**Experimental**

**Plant material and chemicals**

Seeds of *M. pruriens* were collected during the year 2012. Analytical grade methanol, reagent grade gallic acid, Folin-Ciocalteau reagent and sodium carbonate were purchased from Sisco Research Laboratories (SRL), Mumbai, India. DPPH was purchased from Sigma-Aldrich, Bangalore, India. Standard L-DOPA was also purchased from SRL, Mumbai, India. Formic acid was purchased from Merck, Mumbai, India. Deionized water used for HPLC analysis was prepared using a Millipore water purification system (Millipore, Milli-Q, France).

**Preparation of extract**

After removing the immature and damaged seeds, the mature seeds were dried under shade conditions. Further, powder of dried seed (100 mesh) was prepared using an electric grinder (Retsch GmbH, SM100 Comfort, 1500 Watt, Haan, Germany) and powder samples were utilized for preparation of the extracts. For conventional extraction (refluxing), 5 g of powdered plant material was mixed with 50 mL water acidified with 0.1 N hydrochloric acid (pH = 2.6) in a round bottom flask and refluxed for about 5 h at 100°C. Liquid extracts obtained were separated from the

![Figure 2. Comparison of extraction yield of *M. pruriens* seed.](image-url)

Table 1. Extract yield, total phenol, L-DOPA concentration and IC50 value of *M. pruriens* seed.

| Variety       | Extraction technique (Time) | Yielda | Total phenola | L-DOPAb | IC50 (µg/ml) |
|---------------|----------------------------|--------|---------------|----------|--------------|
| Arka Dhanwantri | Reflux (5 h)               | 60.2±1.43 | 5.9±0.03 | 4.4±0.03 | 7.8          |
|               | Sonication (5 min)         | 30.7±1.61 | 3.9±0.11 | 4.7±0.03 | 10.6         |
|               | (10 min)                   | 25.6±0.87 | 4.2±0.09 | 5.4±0.04 | 8.0          |
|               | (15 min)                   | 31.5±0.98 | 5.6±0.04 | 5.0±0.12 | 6.8          |
|               | Microwave (5 min)          | 53.5±0.77 | 12.9±0.1 | 8.2±0.08 | 5.1          |
|               | (10 min)                   | 58.7±1.61 | 23.2±0.05 | 9.1±0.13 | 8.2          |
|               | (15 min)                   | 58.4±0.56 | 9.1±0.11 | 5.4±0.05 | 5.0          |
| Arka Ashwini   | Reflux (5 h)               | 65.7±0.89 | 5.5±0.02 | 4.1±0.05 | 7.6          |
|               | Sonication (5 min)         | 29.0±1.01 | 3.2±0.05 | 2.4±0.03 | 8.5          |
|               | (10 min)                   | 27.7±2.33 | 3.5±0.02 | 3.4±0.02 | 7.4          |
|               | (15 min)                   | 26.8±0.45 | 3.8±0.02 | 4.3±0.02 | 6.4          |
|               | Microwave (5 min)          | 50.6±0.88 | 9.9±0.07 | 6.0±0.01 | 6.0          |
|               | (10 min)                   | 59.6±1.42 | 11.3±0.01 | 7.1±0.07 | 7.6          |
|               | (15 min)                   | 54.0±2.03 | 11.7±0.03 | 7.9±0.07 | 6.2          |
| White          | Reflux (5 h)               | 57.2±2.48 | 6.3±0.01 | 2.9±0.04 | 7.3          |
|               | Sonication (5 min)         | 29.3±1.00 | 4.6±0.07 | 0.8±0.06 | 6.5          |
|               | (10 min)                   | 31.4±0.97 | 4.2±0.06 | 3.6±0.05 | 7.5          |
|               | (15 min)                   | 30.8±1.13 | 5.3±0.06 | 4.4±0.01 | 6.4          |
|               | Microwave (5 min)          | 50.5±2.12 | 9.1±0.13 | 5.4±0.01 | 5.2          |
|               | (10 min)                   | 49.6±2.04 | 7.4±0.01 | 4.7±0.05 | 6.2          |
|               | (15 min)                   | 58.5±0.76 | 13.2±0.01 | 5.1±0.04 | 5.3          |
| Brown          | Reflux (5 h)               | 59.8±0.36 | 7.0±0.09 | 3.7±0.03 | 8.4          |
|               | Sonication (5 min)         | 23.9±1.90 | 5.7±0.06 | 1.6±0.03 | 11.3         |
|               | (10 min)                   | 28.7±1.11 | 5.9±0.09 | 2.5±0.01 | 7.6          |
|               | (15 min)                   | 30.6±1.31 | 5.6±0.04 | 4.7±0.07 | 9.1          |
|               | Microwave (5 min)          | 56.2±2.51 | 10.3±0.01 | 5.7±0.08 | 9.1          |
|               | (10 min)                   | 54.9±0.55 | 8.9±0.07 | 5.7±0.11 | 5.6          |
|               | (15 min)                   | 54.8±0.70 | 7.1±0.10 | 3.9±0.10 | 7.1          |

*a = 3, mean ± standard deviation.

bL-DOPA percentage in seed: extract yield × L-DOPA percentage in extract.
solid residue by vacuum filtration (Filter Discs, Grade-292, Diameter-125 mm, Sartorius, Germany) and concentrated using a vacuum rotary evaporator (Heizbed Hei-VAP, Heidolph, Germany).

For UASE, 5 g of powdered plant material was mixed with 50 mL of acidified water in beaker. Extraction was carried out by placing beaker in an ultrasonic bath (Bandelin Sonorex, Germany, 480 W, 35 kHz) for 5, 10 and 15 min. Water in the ultrasonic bath was circulated at room temperature (25°C) to avoid overheating caused by ultrasound. The supernatant was similarly processed as described in conventional extraction to get dried UASE extract of *M. pruriens*.

Laboratory grade microwave apparatus (1200 W, 2450 MHz, Star Synth, Microwave Synthesis Lab Station, Table 2. Method validation parameters for quantification of L-DOPA by HPLC method.

| Standard | Retention time (min) | Linear range (µg/ml) | LOD (µg/ml) | LOQ (µg/ml) | Equation | $r^2$ |
|----------|----------------------|-----------------------|-------------|-------------|----------|-------|
| L-DOPA   | 4.06±0.042           | 25–500                | 15          | 25          | $Y = 12,000X + 97,300$ | 0.999 |

Table 3. Accuracy and precision (RSD) at three different concentrations for L-DOPA.

| Analyte  | Concentration (µg/ml) | Intra-day | Inter-day |
|----------|-----------------------|-----------|-----------|
| L-DOPA   | 25 (low)              | 0.095     | 1.46      |
|          | 100 (medium)          | 0.128     | 0.62      |
|          | 500 (high)            | 0.885     | 1.26      |

Figure 3. HPLC chromatograms of (a) standard L-DOPA, (b) Arka Dhanwantri (MASE extract), (c) Arka Ashwini (MASE extract), (d) *Mucuna* White (MASE extract) and (e) *Mucuna* Brown (MASE extract).
Milestone Inc., USA) was used for MASE. Powdered plant material (5 g) was mixed with 50 mL of acidified water in a two necked round bottom flask fitted with a condenser and fiber optic sensor for temperature control throughout the experiment. Extraction was carried out by microwave radiation exposure at a power level of 400 W for 5, 10 and 15 min at 60°C. At the end of heating, the flask was left for temperature stabilization. The supernatants were re-centrifuged and concentrated as described earlier. Dried extract samples were kept in an airtight container at 4°C. Standard and working solutions of appropriate dilution of L-DOPA were prepared in acidified water for HPLC method development.

**Determination of total phenolics content (TPC) in extracts**

TPC in the extract samples were determined by Folin-Ciocalteau method using a UV-Visible spectrophotometer (23). Dried extracts were reconstituted in distilled water (1 mg/mL). Folin-Ciocalteau reagent (0.5 mL) was added to extract solution (0.5 mL) and total volume was adjusted to 8.5 mL with distilled water. The tubes were kept at room temperature for 10 min and thereafter 1.5 mL sodium carbonate (20%) was added. The tubes were incubated in water bath at 40°C for 20 min. The intensity of the blue color developed was measured by recording the absorbance at 755 nm using UV-Visible spectrophotometer (Varian, CARY-300 Bio, USA). The reagent blank was also prepared using distilled water. A standard calibration curve was prepared using gallic acid for quantification of the total phenolic in the extract. TPC of the extract samples was expressed as gallic acid equivalent (GAE, mg/g) of the extract.

**Quantification of L-DOPA in the extracts**

The concentration of L-DOPA was quantified in the extract samples using HPLC system consisting of a separation module (Waters 600E) comprising of quaternary pump, an in-line vacuum degasser and a photodiode array detector (Waters 2996). The chromatographic separation was carried out using modified method developed in an isocratic elution mode on Waters Xterra column (C18, 4.6 × 250 mm, 5 µm). The mobile phase was a mixture of methanol (2%, solvent A) and 0.1% formic acid (98%, solvent B). The solvent flow rate was 1.2 mL/min. The injection volume was 20 µL and the column temperature was ambient. The photo diode array detector wavelength was set at 280 nm for the determination of L-DOPA in different extracts of *M. pruriens*. Chromatographic peaks were identified on the basis of matching retention time as well as spectral data. The concentration of L-DOPA was calculated by comparing the integrated peak area with that of a calibration curve prepared using standard L-DOPA.

**Radical scavenging activity using DPPH method and calculation of IC50 concentration**

The radical scavenging activity (RSA) of extracts of *M. pruriens* prepared by refluxing, UASE and MASE methods was evaluated using DPPH assay. Different concentrations of the extracts were taken in test tubes. The
total volume was adjusted to 8.5 mL by addition of methanol. Methanolic solution of DPPH (0.1 mM, 5.0 mL) was added to these tubes and mixed well with a vortex mixer. The tubes were kept at room temperature for 20 min. The blank was prepared as above but without the extract. Methanol was used for the baseline correction. Changes in the absorbance of the extract samples were measured at 517 nm using UV-Visible spectrophotometer. RSA was expressed as the inhibition percentage and calculated using the following formula

\[
RSA, \% = \frac{(\text{Absorbance of blank} – \text{absorbance of sample})}{\text{Absorbance of blank}} \times 100.
\]

The extract concentration corresponding to 50% inhibition (IC\textsubscript{50}) was calculated from the curve of RSA percentage against extract concentration. Ascorbic acid and trolox were used as standards. Each sample was assayed in triplicate for each concentration.

**Statistical analysis**

All the data were analyzed and expressed as mean ± standard deviation of three separate determinations (\(n = 3\)). The statistical analysis was carried out by using Microsoft Excel program.

**Conclusion**

Use of suitable extraction methods will increase versatile utilization of *M. pruriens* seed with high levels of bioactive compounds for the management of chronic diseases like Parkinsonism, diabetes, etc. Results of the present investigation established that the UASE and MASE methods can be viable alternative of conventional extraction methods. However, for industrial application purposes, further investigations are required to develop mathematical model to control and predict the optimization parameters of the extraction process. Green extraction techniques UASE and MASE, besides improving the extract yield and quality also would be beneficial in terms of energy and time consumption. Further, developed HPLC method may be directly utilized for routine screening of species/cultivars/germplasms of *Mucuna* beans for the quantitative determination of L-DOPA. A large number of samples can be screened using this HPLC method as no pre-treatment is required and also the analysis time is less than 10 min.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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