Journal of Pharmaceutical and Medicinal Research 2(1) (2016) 48–51

ISSN: 2455-0280

Contents List available at JACS Directory

Journal of Pharmaceutical and Medicinal Research

journal homepage: www.jacsdirectory.com/jpmr

Phytochemical Analysis and Antioxidant Activity of *Mucuna sanjappae*: A Possible Implementation in the Parkinson's Disease Treatment

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ARTICLE DETAILS

Article history:
Received 01 April 2016
Accepted 11 April 2016
Available online 25 April 2016

Keywords:
Antioxidant
Mucuna sanjappae
Oxidative Stress
Parkinson's Disease

ABSTRACT

The antioxidative potential of *Mucuna sanjappae* seed was evaluated through in vitro assays. The antioxidant activity of water extract in combination with three different in vitro assays viz., 2, 2-diphenyl- 1- picrylhydrazyl (DPPH), *N*, *N*-dimethyl-*p*-phenylendiamine (DMPD) radical scavenging and ferric-reducing antioxidant power (FRAP) assay was evaluated. The total polyphenol contents (TPC) and total flavonoids content (TFC) were also determined. Tannic acid, gallic acid, p-hydroxybenzoic acid and p-coumeric acid were successfully quantified using RP-HPLC-DAD. The strong antioxidant activity of *M. sanjappae* may provide a platform for future drug discoveries and novel treatment strategies in oxidative stress related diseases like Parkinson's disease (PD).

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are free radical entities of normal physiology of the body [1, 2]. Reactive oxygen species includes superoxide anion radicals (O^{-}_{2}), hydroxyl radicals (OH^{-}), non-free radicals species (H_2O_2) and the singlet oxygen (O_2^{*}).Whereas nitric oxide (NO) combines with superoxide to form peroxynitrite which is a strong and versatile reactive nitrogen species [3, 4]. Free radicals can damage cell structures such as carbohydrates, nucleic acids, lipids, proteins and alter their functions adversely [5]. In built antioxidants system of body plays important role in the prevention of damage due to such excited free radicals. This inbuilt system consists of antioxidant enzymes and synthesized antioxidant [5, 6]. An imbalance between oxidants and antioxidant system results in oxidative stress, ultimately in cell impairment and death [7, 8]. Oxidative stress plays an important role in diseases including inflammation, rheumatoid arthritis, cancer, aging, cardiovascular diseases and neurodegenerative diseases [7, 9-12]. Free radicals (ROS) damages cell components including DNA, mitochondria, protein structures and the cell membrane of dopaminergic neurons resulting in functional disruption and cell death which ultimately results in Parkinson's disease [10]. Hence, to fulfill the optimum requirement and to get relief from oxidative damage of cell we have to rely on external source of natural antioxidant.

Mucuna sanjappae (MS) is a new species of genus *mucuna* discovered from Junner area of Western Ghats, Maharashtra, India [13]. It is observed that, local tribal people uses MS beans for various disease treatment including male infertility and PD. L-DOPA (anti-Parkinson's drug) content with nutritional and anti-nutritional component of MS seeds has been reported [14]. The high L-DOPA content (7.3%) in *M. sanjappae* seeds indicate that it is a potential source of anti-Parkinson's drug and will reduce the burden on existing sources of this valuable drug [14]. As we know oxidative stress greatly contributes to the development of PD, study of antioxidant activity in *M. sanjappae* bean is an essential research effort. To this point, the objective of the present research attempt was to evaluate the antioxidant activity of *M. Sanjappae* beans with determination of major phenolics compounds using RP-HPLC-DAD.

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2. Experimental Methods

2.1 Chemicals

All solvents and chemicals were of analytical grade and obtained from local suppliers. Aluminum trichloride, ascorbic acid, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric chloride, Folin-Ciocalteu reagent, potassium phosphate (monobasic and dibasic), sodium carbonate, 2,4,6-tripyridyls-triazine (TPTZ) and trichloroacetic acid were purchased from Sigma Chemical Co., USA. N, N-dimethyl-p-phenylendiamine (DMPD) was purchased from Fluka (Schweiz, Switzerland). Glacial acetic acid (HPLC grade) and methanol (HPLC grade) were purchased from Merck.

2.2 Mucuna sanjappae Seed Water Extract Preparation (MSWE)

The *M. sanjappae* seeds were collected from the Junner area of Western Ghats of India. The fine powder was made and stored in polythene bag until further use. Water was used for the extraction of phytochemicals from the *M. sanjappae* seeds. Appropriate quantity of seed powder was dissolved in water and kept on rotatory shaker for 2 h at 120 rpm. Then it was sonicated for 15 min for the maximum extraction of phytocomponents. The sample was centrifuged at 10,000 rpm for 15 min and supernatant was used for further study.

2.3 Phytochemical Analysis

2.3.1 Determination of Total Phenolics Content (TPC)

The total polyphenol content (TPC) of MS seed water extract (MSWE) was determined spectrophotometrically [15]. In brief, an appropriate volume of the sample (1 mL) was oxidized with 1.8 mL of Folin-Ciocalteu reagent. After 5 min incubation at 25 °C the reaction was neutralized by adding 1.2 mL of 15% sodium carbonate solution and allowed to stand for 90 min at room temperature. The absorbance was measured at 765 nm. Total phenolics value is expressed in terms of mg of gallic acid equivalent (mg GAE g^{-1}) of dry mass.

2.3.2 Determination of Total Flavonoids Content (TFC)

The flavonoids content was determined according to the method described by Chang et al. [16]. 1 mL MSWE sample was mixed with 1.5 mL methanol, 0.1 mL of aluminium chloride (10%) and 0.1 mL of potassium acetate (1 M). Total volume was adjusted to 4.5 mL by the addition of 2.8 mL of D/W. Reaction mixture then incubated for 30 min at room

temperature and absorbance was measured at 415 nm. This was compared to the standard curve of quercetin concentrations and expressed as milligram of quercetin equivalents per gram (mg QUE g^{-1}) of dry weight.

2.4 Antioxidant Capacity of MSCP

Antioxidant capacity of extract was determined by following different assays.

2.4.1 DPPH Free Radical Scavenging Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging potential of the MSWE was measured [17] with slight modification [18]. The stock reagent solution was prepared by dissolving 24 mg of DPPH in 100 mL methanol and stored at -20 °C until required. The working solution was obtained by mixing 10 mL of stock solution with 45 mL methanol to obtain an absorbance value of 1.1 ± 0.02 at 515 nm, using a spectrophotometer. The different volumes of MSWE respective extract (100 – 500 µL) were allowed to react with DPPH solution in the final reaction volume of 3 mL. The mixture was shaken vigorously and allowed to stand in the dark at room temperature. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 517 nm against methanol. The absorbance of control sample (without extract) was also analyzed and the results were expressed as radical scavenging capacity (% RSC). Ascorbic acid was used as a standard (20 - 100 µM). The DPPH radical scavenging capacity was calculated using the following equation.

$$\% \text{ RSC} = A_{(control)} - A_{(sample)}/A_{(control)} \times 100$$

2.4.2 DMPD Scavenging Capacity

DMPD (N, N-dimethyl-p-phenylendiamine) assay was performed according to method described by Fogliano et al [19]. This assay is based on the capacity of the extract to inhibit the $DMPD^{\bullet+}$ radical cation. For this aim, 100 mM DMPD solution was prepared by dissolving 209 mg DMPD in 10 mL distilled water. One mL of this solution was added to100 mL 0.1 M acetate buffer (pH 5.2). $DMPD^{\bullet+}$ was obtained by the addition of 0.2 mL of a 0.05 M ferric chloride solution. Different volumes of MSWE (10 - 50 µL) were added to 2.0 mL of $DMPD^{\bullet+}$ solution and the total volume was adjusted with distilled water to 3.0 mL and incubated for 10 min. The absorbance was measured at 505 nm. Buffer solution was used as blank and instead of sample distilled water was used for control. The scavenging activity was compared with standard ascorbic acid (20 – 100 µM). The $DMPD^{\bullet+}$ scavenging capacity was calculated using the following equation.

$$DMPD^{\bullet+} \text{ scavenging capacity (\%)} = A_{control} - A_{sample}/A_{control} \times 100$$

2.4.3 FRAP (Ferric Reducing/Antioxidant Power) Assay

The FRAP assay of MSWE was also carried out [20]. Briefly, the working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$ in 10:1:1 ratio prior to use and heated to 37 °C in an hot water bath for 10 min. The different volumes of MSWE (10 – 300 µL) were allowed to react with 3.0 mL of the FRAP reagent. The final volume of the reaction mixture was made up to 4.0 mL with distilled water. The reaction mixture was kept in dark at room temperature for 30 min. The absorbance of the colored product (ferrous tripyridyltriazine complex) was then recorded at 593 nm using UV-visible spectrophotometer. The activity was compared with standard ascorbic acid (50 – 250 µM). A higher absorbance reading indicated a higher reducing power.

2.5 RP-HPLC-DAD Determination of Phenolics in M. sanjappae Seeds

The analysis of phenolic compounds in MSWE was determined using a high-performance liquid chromatography system on a waters HPLC (Model 2487), using a hypersil C18 reversed-phase column 25 cm with 5 µm particle size with 1 mL min^{-1} flow rate. The mobile phase consisted of 25% methanol in 1% acetic acid and injection volume was 20 µL. The phenolic acids were identified at 280 nm by comparing the retention time of sample chromatographic peaks with those of authentic standards using the same HPLC operating conditions.

3. Results and Discussion

3.1 Total Polyphenol and Flavonoid Content

Phenolics and flavonoids are natural antioxidant substances having capability of scavenging reactive oxygen and nitrogen species, reducing the risk of oxidative stress related diseases like cancer and protect biological system [21]. The MS seed showed 75.27 ± 4.71 mg GAE g^{-1} of TPC content. The flavonoids content was 472 ± 9.51 mg QAE g^{-1}which suggests that MS beans might be beneficial for reducing oxidative stress and related disorders like diabetes and Parkinsonism. The flavonoids have been reported for their anti-Parkinson's activity on 6-OHDA-induced experimental Parkinsonism model [22].

3.2 Antioxidant Activity of MSWE

The in vitro assays are based on the ability of scavenging synthetic free radicals, using a variety of radical creating systems. However, evaluation of total antioxidant capacity of sample could not be performed correctly by any single method due to the multifarious nature of phytochemicals [23]. DPPH, $DMPD^{\bullet+}$ and FRAP radical scavenging methods are common spectrophotometric procedures for determining antioxidant capacities of plant based drugs and food. Different solvents are used for the extraction of interested phytocomponents according to specific purpose. Among the different solvents, water is the feasible and cost effective solvent for the study and also used in the herbal drug preparation. Hence, study was undertaken to evaluate antioxidant activity of MS bean water extract (MSWE).

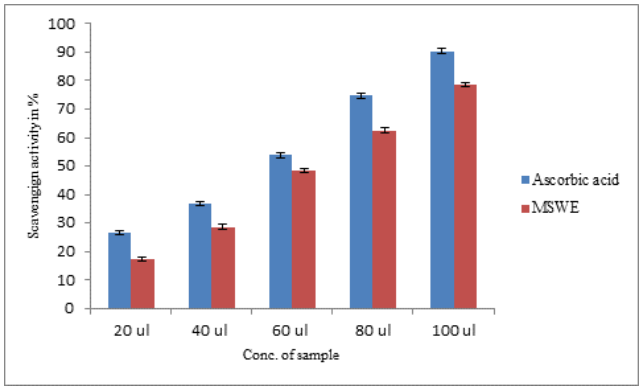

Fig. 1a DPPH radical scavenging activity of *M. sanjappae* water extract (MSWE) (conc. 1 mg/mL) in comparison with ascorbic acid (conc. 1 mM) expressed in % (± SEM)

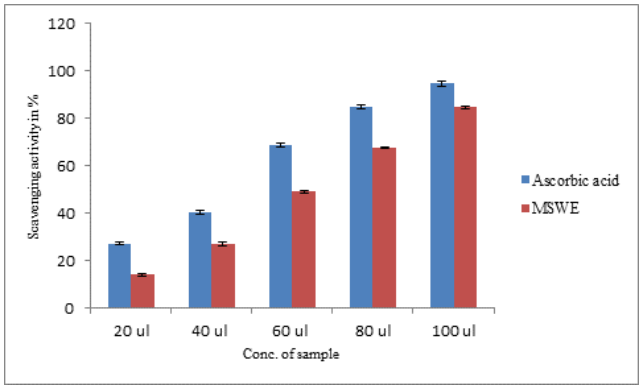

Fig. 1b DPPD radical scavenging activity of *M. sanjappae* water extract (MSWE) (conc. 1 mg/mL) in comparison with ascorbic acid (conc. 1 mM) expressed in % (± SEM)

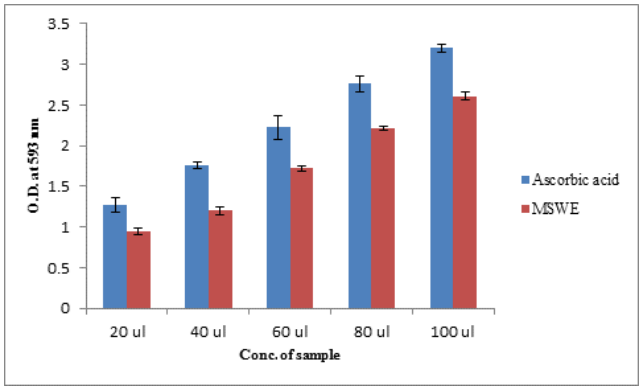

Fig. 1c FRAP radical scavenging activity of *M. sanjappae* water extract (MSWE) (conc. 1 mg/mL) in comparison with ascorbic acid (conc. 1 mM) expressed in O.D. at 593 nm (± SEM)

3.2.1 DPPH Free Radical Scavenging Activity

This method is based on the reduction of DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. Dark color of DPPH radical solution becomes lighter and absorbance of solution becomes lower in the presence of an antioxidant compound [24]. The MSWE extract was capable of scavenging DPPH radicals in a concentration dependent manner. It showed 17.29 ± 0.67% and 78.57 ± 0.812% DPPH scavenging capacity at the dose of 20 and 100 µL (20 and 100 µg) respectively. Standard ascorbic acid showed 26.433 ± 0.759% and 90.32 ± 0.766% DPPH radical scavenging capacity at the 20 µL and 100 µL (20 and 100 µM) concentration respectively. The result confirmed that, MSWE possesses strong antioxidant capacity viz., scavenging unstable DPPH free radicals (Fig. 1a).

3.2.2 DMPD Scavenging Activity

This method is based on the ability of the antioxidant compounds to transfer a hydrogen atom to the colored radical $DMPD^{\bullet+}$ turning it into an uncolored $DMPD^{+}$ compound [25]. The assay has some advantages as high stability end point, quick reaction time, cost effectiveness and is less cumbersome [28]. The DMPD scavenging capacity of MSWE extract is shown in Fig. 1b. It increased significantly in a concentration dependent manner, with 13.84 ± 0.537% at 20 µL (20 µg) while 84.67 ± 1.03% antiradical activity at 100 µL (100 µg) whereas standard ascorbic acid presented at 27.18 ± 0.529 and 94.65 ± 0.56 at the 50 and 250 µL (50 µM and 250 µM) concentration respectively.

3.2.3 FRAP Activity

Ferric reducing antioxidant power assay is a simple and quick assay routinely carried out for the determination of antioxidant activity [2] in terms of optical density. MS seed sample showed 0.954 ± 0.041 and 2.61 ± 0.043 O.D. at 593 nm at the concentration of 20 and 100 µL (20 and 100 µg) respectively. Ascorbic acid exhibited 1.26 ± 0.09 at 50 µM (50 µg) and 3.20 ± 0.04 at 250 µM (250 µg) concentration (Fig. 1c). FRAP activity also proved the efficiency of MSWE to scavenge free radical generated in vitro.

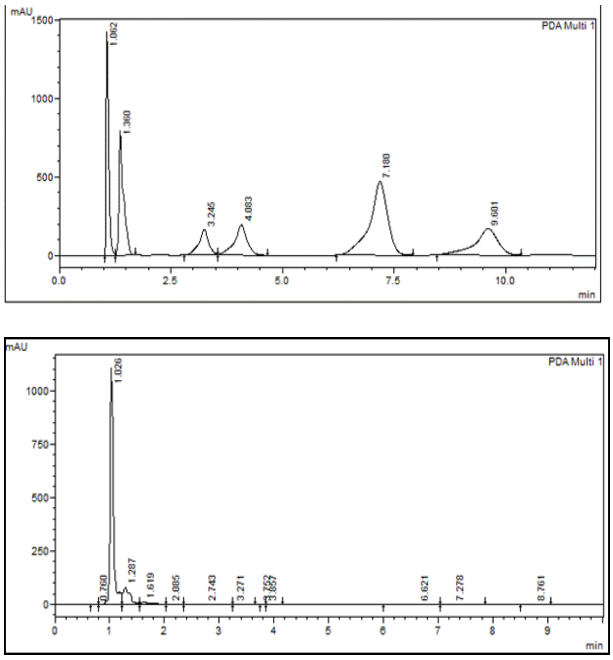

Fig. 2 HPLC chromatograms of a standard mixture of phenolic acids (a) and profiles of *M. sanjappae* water extract (b). Peak 1, tannic acid; 2, gallic acid; 3, p-hydroxybenzoic acid; 4, vanillic acid; 5, p- coumaric acid; and 6, ferulic acid

3.4 RP-HPLC-DAD Analysis of Phenolics

Phenolics are the major group of compounds responsible for antioxidant activity of plant sample. The major phenolics were quantified using HPLC and found that, MS bean possesses tannic acid (143.34 ± 1.14 mg/gm), gallic acid (12.97 ± 0.5 mg/gm), *p*-hydroxybenzoic acid (0.44 ± 0.01 mg/g) and p-coumeric acid (1.37 ± 0.04 mg/g) (Fig. 2). Tannic acid has several health benefits such as anti-carcinogenic, antioxidant, anti-mutagenic, antimicrobial, antiallergic, anti-inflammatory, stopping bleeding and to alleviate hypertensive effect [26-29]. Gallic acid has been studied for its anti-PD potential [30]. p-hydroxybenzoic acids is widely used as a preservative in food and pharmaceutical industries [31]. p-coumaric acid is a kind of hydroxycinnamic acid [32] used as a precursor for the production of important aromatic natural products [33].

4. Conclusion

The interest of people in herbal drugs is continuously increasing because of long term efficacy with less secondary complication. Antioxidant potential of such herbal drugs is an essential factor for their extensive usefulness. The present study is the first report on antioxidant activity of *M. sanjappae*. Oxidative stress generated cell degeneration is one of the steps during progression of PD as well as any other stress related disorder. Hence, natural medicine having potential of releasing oxidative stress will be the important way of disease management. The higher content of L-DOPA in *M. sanjappae* seeds suggests its exploitation potential as an anti-PD drug. The additional properties like antioxidant activity will add some benefits to this plant species. Our results clearly indicate the antioxidant potential of *M. sanjappae* and its future usefulness in herbal medicines especially for providing protection in cellular oxidative stress and related diseases. Further animal and cell line study is necessary to evaluate the antioxidant and anti-Parkinson's potential of MS beans.

Acknowledgement

Prof. Jyoti P. Jadhav sincerely thanks Department of Biotechnology, Government of India for "Interdisciplinary Programme of Life Sciences for Advanced Research and Education (IPLS- Reference No: BT/PR4572/INF/22/147/2012)". Ravishankar R. Patil thanks to IPLS for providing fellowship; Vishwas A. Bapat thanks Indian National Science Academy, New Delhi for senior scientist fellowship.

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Cite this Article as: R.R. Patil, M.R. Rane, V.A. Bapat, J.P. Jadhav, Phytochemical analysis and antioxidant activity of *Mucuna sanjappae*: A possible implementation in the Parkinson's disease treatment, J. Pharm. Med. Res. 2(1) (2016) 48–51.