FREE RADICAL SCAVENGING ACTIVITY OF *POLYGALA CHINENSIS* LINN.

Shailendra Gurav*, Nilambari Deshkar, Vijay Gulkari, Nandkishore Duragkar [a], Arun Patil

Department of Pharmaceutical Sciences, Nagpur University, Amaravati Road, Campus, Nagpur 440 033
[a] Sharad Pawar College of Pharmacy, Wanadongari, Hingna Road, Nagpur

Summary

The purpose of this work comprises the assessment of free radical scavenging activity of alcoholic extract of *Polygala chinensis* Linn. (Polygalaceae) by different analytical methods like 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay, nitric oxide scavenging assay and hydrogen peroxide scavenging assay. The alcoholic extract of *Polygala chinensis* showed concentration dependent free radical scavenging activity in all models. IC_{50} was found to be 56.09 µg/ml, 47.63 µg/ml and 41.003 µg/ml in DPPH assay, NO radical scavenging assay and H_2O_2 scavenging assay respectively. Total phenolic content and total flavonoids were evaluated according to Folin–Ciocalteu reagent and colorimetric method respectively which revealed a high amount of polyphenols (88.2±9.3 mg/100gm) as well as flavonoids 36.7±3.9 mg/100gm, suggesting a possible role of these compounds in the antioxidant properties.
Keywords: *Polygala chinensis;* free radical scavenging activity; DPPH radical assay; nitric oxide radical assay; hydrogen peroxide scavenging assay

***Corresponding author:**
Shailendra S. Gurav,
Department of Pharmaceutical Sciences,
R.T.M. Nagpur University, Nagpur- 440033
Maharashtra (India)
Tel.: +919823432948; fax: +917122500355
E-mail: *shailugurav@rediffmail.com*

Introduction

Polygala chinensis Linn. (Polygalaceae) is also known as *Polygala arvensis* (1). It is distributed throughout India upto 5,000 ft. in tropical Asia and Australia. In Chota Nagpur, it is traditionally used in the treatment of fever and dizziness (2). Aqueous extract is used as galactogogue (3). It contains various useful phytoconstituents such as flavonoids, flavone glycosides, lactonic lignans and saponins (4-8). It is also used in treatment of general debility, fever and liver dysfunctions. Hepatoprotective activity of ethanolic extract of *P. chinensis* has proved in our lab by Deshkar and et. al. (9).

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals (10). Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (11). The most common reactive oxygen species (ROS) include superoxide ($0_2^{\cdot}$) anion, hydrogen peroxide (H_20_2), peroxyl (ROO^{-}) radicals, and reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^{\cdot}$). ROS have been implicated in over a hundreds of disease states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (12). In treatment of these diseases, antioxidant therapy has gained an immense importance.

Antioxidants are able to give an electron to the free radicals and thus eliminating the adverse effects by stabilizing them. Naturally occurring antioxidants in leafy vegetables and seeds, such as ascorbic acid, vitamin E and phenolic compounds possess the ability to reduce the oxidative damage associated with many diseases including cancer, cardiovascular disease, cataract, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing (13). In addition to this, free radical scavengers such as vitamin E, β-carotene and ascorbic acid acts as secondary defense against reactive oxygen species (14). As in the plant kingdom numerous crude extracts and pure natural compounds were previously reported to have antioxidant properties; so many researchers have focused on natural antioxidants. Plants containing flavonoids and phenolics have been reported to possess strong antioxidant properties (15-18). Hence, in the present study, the *P. chinensis* was screened for *in-vitro* antioxidant properties using standard procedures.

Methods

Plant material:

P. chinensis was collected from the local area in the month of September 2006 and authenticated by a taxonomist, Department of Botany, Nagpur University, Nagpur. A voucher specimen (voucher no. 5881/1) has been preserved and available with the Department of Botany, Nagpur University, Nagpur.

Preparation of extracts:
The whole plant was washed, shade dried and powdered. The weighed powder (150 g) was defatted with petroleum ether (60-80°C) and then extracted with ethanol using soxhlet apparatus (35 cycles each). The extract was concentrated for further studies at reduced pressure and temperature in a rotary evaporator. The defatted alcoholic extract was 13.29% w/w of the starting material. Alcoholic extract was tested for presence of secondary metabolites by different phytochemical tests (19).

Drugs and Chemicals:
Ascorbic acid, DPPH, Gallic acid, Quercetin and Folin–Ciocalteu reagent were obtained from the Sigma chemicals, USA. Ethanol was purchased from Ranbaxy laboratories Ltd. Punjab. All other chemicals used were of analytical grade and obtained from common sources.

Total phenolic content:
Total phenolic content was determined according to a protocol similar to that of Kahkonen MP and et.al. (17). *P. chinensis* alcoholic extract (10mg) was mixed with 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin–Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5% Na_2CO_3 was added. Thereafter, it was thoroughly mixed and placed in the dark for 1 h and the absorbance was measured at 725 nm using UV/Visible spectrophotometer. The amount of total polyphenols was calculated as a gallic acid equivalent from standard curve of gallic acid solutions (covering the concentration range between 10- 1000 µg/ml), and expressed as mg/100g of extract.

Determination of Total flavonoids:
Aluminum chloride colorimetric method was used for flavonoids determination (20). 10mg of test extract was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2ml of 1 M potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with UV/Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at various concentrations in methanol. The concentration of flavonoids was expressed in terms of mg/100g of extract.

Free radical scavenging activity:

DPPH radical scavenging assay
The free radical scavenging activity of alcoholic extract of *P. chinensis* was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH (21). 0.1mM solution of DPPH (1, 1-diphenyl-2-picryl-hydrazyl) in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution of *P. chinensis* in double distilled water at different concentrations (10-100 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of DPPH radicals by 50%. Ascorbic acid was used as reference standard.

Nitric oxide radical scavenging assay

The interaction of alcoholic extract of *P. chinensis* with nitric oxide was assessed by the nitrite detection method (22-24). Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside (5 mM) in phosphate-buffered saline (PBS) was mixed with 3.0ml of different concentrations (10 – 320 µg/ml) of extracts dissolved in the suitable solvent system and incubated at 25°C for 150 min. The samples from the above were reacted with Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. The same reaction mixture without the extract but with equivalent quantity of distilled water served as control. Ascorbic acid was used as reference standard.

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging activity of the extract was estimated by replacement titration (25). Aliquot of 1.0 ml of 0.1 mM H_2O_2 and 1.0 ml of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2 M H_2SO_4 and 7.0 ml of 1.8 M KI. The mixed solution was titrated with 5.09 mM NaS_2O_3 until yellow color disappeared. Percentage of scavenging of hydrogen peroxide was calculated as:

$$\% \text{ Inhibition} = (V_0 - V_1) / V_0 \text{ X } 100$$

Where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_1 was the volume of NaS_2O_3 solution used in the presence of the extract.

Statistical analysis:

Tests were carried out in triplicate for all experiments. The amount of extract needed to inhibit free radicals concentration by 50% i.e. IC_{50} was graphically determined by a linear regression method using MS - Windows based GRAPHPAD INSTAT (version 3) software. Results were expressed as mean± standard deviation.

Results and Discussion

Synthetic antioxidants, such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA), have restricted use in foods as they are suspected to be carcinogenic. Therefore, the importance of search for natural antioxidants has greatly increased in the recent years (26).

Phytochemical screening of extract showed presence of saponin, coumarin, flavonoid and lignan. Phytochemical analysis of alcoholic extract of *P. chinensis* contain rich source of flavonoids 36.7±3.9 mg/100gm and polyphenol 88.2±9.3 mg/100gm. Flavonoids and polyphenols have been proved to possess antioxidant activities (27, 28, 18) and have been used for the prevention and cure of various diseases which is mainly associated with free radicals (29, 30).

Inhibition of DPPH radical

DPPH is a stable free radical at room temperature and often used to evaluate the antioxidant activity of several natural compounds. It accepts an electron or hydrogen radical to become a stable diamagnetic molecule (21). The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517nm, which is induced by antioxidant. Antioxidant on interaction with DPPH, either transfer electron or hydrogen atom to DPPH or thus neutralize its free radical character. As this electron becomes paired in the presence of a free radical scavenger, the absorption vanishes and the resulting decolourisation is stoichiometric with respect to the number of electrons taken up. As shown in Table 1 *P. chinensis* exhibited concentration dependent scavenging activity by inhibiting DPPH radicals generated. The IC_{50} of alcoholic extract of *P. chinensis* was 56.09 µg/ml.

Inhibition of nitric oxide radical

Nitric oxide is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (27). Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states (31). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrites (22-24). Alcoholic extract of *P. chinensis* moderately inhibited nitric oxide in dose dependent manner (Table 1) with the IC_{50} 47.63 µg/ml. Ascorbic acid was used as standard.

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe_2+ and possibly Cu_2+ ions to form hydroxyl radical and this may be the origin of many of its toxic effects (32). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. As shown in Table 1, *P. chinensis* extract demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with the IC_{50} of 41.03 µg/ml.

Conclusion

Phytochemical screening of extract showed presence of saponin, coumarin, flavonoid and lignan. As results shows that it's a rich source of flavonoids and polyphenols which leads to conclusion that high soluble phenolics as well as flavonoids in the *P. chinensis* extract could be taken into account for the strong free radical scavenging activity in all used assay. Therefore, it is suggested that further work should be performed on the isolation and identification of the antioxidant components in *P. chinensis.*

Table 1- Radical Scavenging activity of alcoholic extract of *Polygala chinensis* at different concentrations

Concentration (µg/ml)	DPPH radical scavenging %	NO radical scavenging %	H_2O_2 scavenging
10	12.20±1.164	12.38±0.198	25.45±3.15
20	23.15±1.035	33.46±1.085	45.56±2.86
50	43.11±2.142	64.67±1.313	58.18±2.69
80	75.12±1.092	74.72±1.692	69.09±3.15
100	80.33±1.137	80.48±1.919	81.82±2.87
IC_{50} (µg/ml)	56.09 (0.9909)*	47.63 (0.9479)*	41.00(0.9668) *
IC_{50} (µg/ml) of Ascorbic acid	15.0 (0.963)*	22.20 (0.9450)*	18.03(0.9721) *

Here n=3 and values are expressed as Mean ± S.D., r*- regression coefficient

Acknowledgement

Authors are thankful to the Head, Department of Botany, Nagpur University, Nagpur for their help in identification and authentication of plant and also grateful to AICTE for providing grants to present research work.

References

1. Ugemuge NR. Flora of Nagpur District, Shree Prakashan, Nagpur, 1986, p. 59.
2. Kirtikar KR, Basu BD. Indian Medicinal Plants, International Book Distributors, Dehradun, 1956, p. 263.
3. Asolkar LV, Kakkar KK, Chake OJ. Second Supplement to Glossary of Indian Medicinal Plants with Active Principles, CSIR: New Delhi, 1992, India.

4. Ghosal S, Chouhan RPS, Srivastava RS. Structure of chinensin: a new lignan lactone from *Polygala chinensis*. Phytochemistry 1974; 13: 2281-2284.
5. Ghosal S, Chouhan RPS, Srivastava RS. Two new aryl napthalide lignans from *Polygala chinensis.* Phytochemistry 1974; 13: 1933-1936.
6. Ghosal S, Chouhan RPS, Srivastava RS. Lactonic lignans of *Polygala chinensis.* Phytochemistry 1973; 12: 2550-2551.
7. Rao MS, Rao PS, Kumar JK, Raman NV. A rare flavonol glycoside from *Polygala chinensis*. Biochemical Systematics and Ecology 2003; 31 (6): 635-636.
8. Rao MS, Raman MV. A novel flavonoid from *Polygala Chinensis*. Biochemical Systematics and Ecology 2004; 32 (4): 447-448.
9. Deshkar N, Itankar P, Duragkar N, Patil A. Hepatoprotective activity of *Polygala Chinensis* in carbon tetrachloride induced liver damage in rats. Indian Journal of Natural Products 2006; 22 (4): 21-24.
10. Gutteridgde JMC. Free radicals in disease processes: A complication of cause and consequence. Free Radic. Res. Comm. 1995; 19: 141- 158.
11. Tiwari A. Imbalance in antioxidant defence and human diseases: Multiple approach of natural antioxidants therapy. Curr. sci. 2001; 81: 1179-1187.
12. Joyce DA. Oxygen radicals in disease. Adv. Drug Reac. Bull. 1987; 127: 476-79.
13. Pietta P, Simonetti P, Mauri P. Antioxidant activity of selected medicinal plants. J Agric Food Chem 1998; 46: 4487–4490.
14. Lee KG, Mitchell AE, Shibamoto T. Determination of antioxidant properties of aroma extracts from various beans. J Agric Food Chem 2000; 48: 4817–4820.

15. Raj KJ, Shalini K. Flavonoids—a review of biological activities. Indian Drugs 1999; 36: 668–676.
16. Miller AL. Antioxidant flavonoids: structure,function and clinical usage. Alt. Med. Rev. 1996; 1: 103-111.
17. Kahkonen MP, et.al. Antioxidant activity of plant extract containing phenolic compounds. Journal of Agricultural and Food Chemistry 1999; 47: 3954–3962.
18. Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruomab OI, Bahorun T. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. Mutation Research 2005; 579: 200–213.
19. Kokate CK. Practical Pharmacognosy, Vallabh Prakashan, Delhi; 1992, p. 177-180.
20. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anala. 2002; 10: 178-182.
21. Blosi MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 181: 1199–1200.
22. Green LC, Wagner DA, Glogowski J. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem, 1982; 126: 131-138.
23. Marcocci L, Maguire JJ, Droy-Lefaix MT. The nitric oxide-scavenging properties of *Ginkgo biloba* extract EGb 761. Biochem Biophys Res Commun 1994a; 15: 748–755.
24. Marcocci L, Packer L, Droy-Lefaix MT. Antioxidant action of *Ginkgo biloba* extract EGb 761. Meth. Enzymol. 1994b; 234: 462–475.
25. Zhang XY. Principles of Chemical Analysis. Beijing, China Science Press., 2000, p. 275-276.

26. Jayaprakasha GK, Selvi T, Sakariah KK. Antibacterial and. antioxidant activities of grape (*Vitis vinifera*) seed extract. Food Res Int. 2003; 36: 117–122.
27. Hagerman AE, et al. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agric. and Food Chem. 1998; 46: 1887-1892.
28. Kahkonen MP, Hopia AI, Heinonen M. Berry phenolics and their antioxidant activity. Journal of Agricultural and Food Chemistry 2001; 49: 4076–4082.
29. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem. Pharmacol. 1983; 30: 1141-1148.
30. Middleton E, Kandaswamy C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000; 52: 673–751.
31. Moncada A, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 1991; 43: 109-142.
32. Miller MJ, Sadowska-krowicka H, Chotinaruemol S, Kakkis JL, Clark DA. Amelioration of chronic ileitis by nitric oxide synthase inhibition. J. Pharmac. and Experi.Thera. 1993; 264: 11-16.