Research Article

Studies on Antioxidant Activity of Plants Collected near Indian Oil Gas Plant, Salem, Tamil Nadu, India.

Krishnaveni Marimuthu*, Silpavathi G[1], Silambarasan V[1], Senthil kumar R[1], Sabari M[1], Eswari V[1]
*Assistant Professor, Department of Biochemistry, School of Bio-sciences, Periyar University, Salem, Tamil Nadu, India.
[1]M.Sc Students, Department of Biochemistry, School of Bio-sciences, Periyar University, Salem, Tamil Nadu, India.
***Corresponding author's E-mail:** krishnavenim2011@gmail.com

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ABSTRACT

Plants are one among the five big living things. Plants release oxygen, a by-product of photosynthesis and are helpful in storing carbon, carbon dioxide from burning fossil fuels and also regulates water cycle, purifies the water of the planet. Considering the significance of plants, it was decided to study the antioxidant activities, secondary metabolites for all the plants selected. Various antioxidant activities was tested like phosphomolybdenum, reducing power, nitric oxide scavenging, metal ion chelating, hydrogen peroxide scavenging assay. Among the different tests used for the assessment, *Ficus benghalensis* showed highest activity in total antioxidant assay performed. *Cicca acida* showed highest activity with nitric oxide scavenging activity, *Artocarpus heterophyllus* for metal chelating activity, *Ficus religiosa* showed highest activity of hydrogen peroxide scavenging activity. *Wrightia tinctoria* contained phenolic content in highest level. Similarly, *Eucalyptus tereticornis* had highest flavonoid content. Whereas, moderate with rest of the plants studied. Very few plants showed low levels of antioxidant, secondary metabolites.

Keywords: Antioxidants, Assays, Indian oil gas plant, Plants.

INTRODUCTION

Nearly, only 5% of the oxygen is concentrated to free radicals - superoxide, hydrogen peroxide, hydroxyl, nitric oxide radicals called as reactive oxygen species, which causes an irreversible damage to components of cells. All medicinal plants are reported for their antioxidant activity. The antioxidant properties of the plants are exhibited mainly by secondary metabolites present in the plant. Antioxidants are otherwise called as scavengers, that are able to scavenge the singlet oxygen species, a major supporter for disease. Plants synthesize several new antioxidants to regulate the oxidative stress induced by sunlight and oxygen and also various other environmental stress. Hence, the present work was initiated to study the antioxidant capacity for the selected plants by analyzing different methods and their secondary metabolites was also assessed for *Tectona grandis, Ficus religiosa, Millettia pinnata*, *Psidium guajava*, *Eucalyptus tereticornis*, *Tamarindus indica, Azadirachta indica, Santalum album*, *Casuarina equisetifolia*, *Annona squamosa*, *Cicca acida*, *Wrightia tinctoria*, *Artocarpus heterophyllus*, *Polyalthia longifolia*, *Ficus benghalensis.*

MATERIALS AND METHODS

Leaf Sample Collection

For the present study, fresh leaves from each plant was collected from the experimental site near Indian Oil Gas Plant located at Karupur-Panangadu, Salem, Tamil Nadu, India during the month of December 2014–January 2015. Common plants identified were selected from the study areas. All the selected plants were identified by Dr. A. Balasubramanian and also by comparing with book named Dictionary of Medicinal Plants written by Dr. A. Balasubramanian, Executive Director, ABS Botanical garden, Salem, Tamil Nadu, India.

Extract Preparation

Fresh leaves were used according to the standard prescribed methods adopted. 100mg of fresh leaves was used for extraction. 1ml of distilled water was used for extraction and 0.1ml of clear extract was used for the each experiment assessed.

Quantitative Assays

Assay of Antioxidants

Total antioxidant activity by phosphomolybdenum complex method

0.1ml of extract was mixed with 4ml of reagent solution containing 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. The contents in the tube was incubated in a water bath at 95°C for 90minutes. After the samples had been cooled to RT, the absorbance of mixture was measured at 695nm using UV Visible spectrophotometer. Standard calibration plot was prepared using ascorbic acid.[1]

Reducing Power Assay

0.1ml of plant extract was mixed with 1ml of phosphate buffer (0.2M, pH6.6) and 1% Potassium ferricyanide, shaken well and incubated at 50°C for 20minutes. After incubation, 1ml TCA (10%) was added to stop the reaction. It was centrifuged at 3000rpm for 10minutes. To 1.5ml of supernatant, 1.5ml of distilled water add 0.1ml ferric chloride (0.1%) and the tubes were incubated for

10minutes, the absorbance was read at 700nm using UV Visible spectrophotometer. Standard calibration curve was plotted using ascorbic acid.[2]

Nitric Oxide Scavenging Activity

To 0.1ml of extract, 2ml of 10mM sodium nitroprusside, 0.5ml of phosphate buffered saline 1M was added and then incubated at 25°C for 150minutes. After incubation, 1ml of sulphanilic acid reagent (0.33%), 1ml of naphthylene diamine dihydrochloride (1%) was added and mixed, allowed to stand for 30minutes. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions can be estimated by the use of Griess illsovery reaction at 540nm.[3,4] Quercetin was used as a standard.

Metal Ion Chelating Activity

To 0.1ml of extract add 2.16ml of distilled water, 80µl of 2mM ferric chloride. The reaction was initiated by the addition of 160µl of ferrozine. The contents in the tube was mixed well and allowed to stand for 10minutes at room temperature. After incubation the absorbance was read at 562nm using UV Visible spectrophotometer. The calibration plot was drawn using ascorbic acid as a standard.[5]

Hydrogen Peroxide Scavenging Activity

To 0.1ml of extract add 0.6ml hydrogen peroxide solution (0.6ml, 40mM). The absorbance of hydrogen peroxide at 230nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. A solution of hydrogen peroxide (40 mM) was prepared in phosphate solution. The percentage of hydrogen peroxide scavenging activity exhibited by the extracts and standard compounds was calculated as follows: % Scavenged [H_2O_2] = [(Ao – A1)/Ao] × 100 where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract.[8,6]

Secondary Metabolites

Total Phenolics

To 0.1ml of extract, added 2.8ml of 10% sodium carbonate, 0.1ml of 2N folin ciocalteu phenol reagent. After 40minutes incubation, the color developed was read at 725nm using UV- Spectrophotometer. Total phenolic contents calculated was expressed as mg of gallic acid equivalents/g of sample using standard calibration curve constructed.[7]

Total Flavonoids

0.1ml of plant extract was mixed with 1.5ml of methanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It remained at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415nm using UV/Visible spectrophotometer. Total flavonoid content was calculated from a calibration curve obtained using quercetin as a standard.[8,9]

Statistical Analysis

Each experiment was carried out in triplicate and the results are given as the Mean ± Standard deviation.

The Mean and Standard deviation (S) was calculated by using the following formula:

$$Mean = \frac{Sum\ of\ x\ values}{n\ (Number\ of\ values)},$$

$$s = \frac{\sqrt{\sum(X-M)^2}}{n-1}$$

RESULTS AND DISCUSSION

The results of antioxidant analysis is shown in Table 1 and the results of secondary metabolites are shown in Table 2.

Posphomolybdenum Activity

The Posphomolybdenum activity was high with *Ficus benghalensis* 5.10 ± 0.17, *Casuarina equisetifolia* 5.00 ± 0.86, whereas, moderate amount was observed with *Cicca acida* 4.86 ± 0.75, *Annona squamosa* 4.63 ± 1.15, *Polyalthia longifolia* 4.47 ± 0.63, *Artocarpus heterophyllus* 4.13 ± 0.11, *Millettia pinnatta* 4.03 ± 1.50, *Azadirachta indica* 3.83 ± 1.15, *Eucalyptus tereticornis* 3.40 ± 0.51, *Wrightia tinctoria* 3.20 ± 0.69, *Santalum album* 3.16 ± 0.92, *Tamarindus indica* 3.00 ± 0.69. While, the activity was low with *Psidium guajava* 2.60 ± 0.51, *Ficus religiosa* 2.53 ± 0.63, *Tectona grandis* 1.13 ± 0.11. Similar result was reported by Krishnaveni for *Azadirachta indica*[14] *Annona squamosa, Casuarina equisetifolia, Polyalthia longifolia*[15,16].

Reducing Power Assay

Only moderate amount of reducing power activity was observed with almost all the plants studied. Their reducing power activities are shown as follows: *Artocarpus heterophyllus* 4.20 ± 0.69, *Tamarindus indica* 3.90 ± 0.30, *Ficus benghalensis* 3.26 ± 0.92, *Annona squamosa* 3.23 ± 0.80. The reducing power activity was low with *Azadirachta indica* 1.30 ± 0.00, *Ficus religiosa* 1.73 ± 0.37. Similar result was reported by Krishnaveni for *Casuarina equisetifolia,*[10] *Tectona grandis,*[12] *Tamarindus indica.*[14,17].

Nitric Oxide Scavenging Assay

Nitric oxide scavenging activity was high with *Cicca acida* 5.13 ± 0.11, *Casuarina equisetifolia* 4.38 ± 1.50, *Ficus religiosa* 4.30 ± 0.00. Moderate amount was observed with remaining plants studied such as *Millettia pinnatta* 3.98 ± 1.15, *Eucalyptus tereticornis* 3.68 ± 1.41, *Wrightia tinctoria* 3.67 ± 1.87, *Santalum album* 3.46 ± 1.01, *Azadirachta indica* 3.41 ± 0.14, *Tamarindus indica* 3.27 ± 1.78, *Psidium guajava* 2.97 ± 1.52, *Tectona grandis* 2.73 ± 0.28, *Annona squamosa* 2.20 ± 0.77, *Artocarpus heterophyllus* 2.17 ± 0.56, *Polyalthia longifolia* 2.14 ±

0.53. Similar result was reported by Krishnaveni for *Annona squamosa,*[13] *Tamarindus indica.*[17].

Metal Ion Chelating Activity

Ion chelating activity was high with *Santalum album* 6.83 ± 1.67, *Artocarpus heterophyllus* 6.13 ± 2.54, *Psidium guajava* 6.10 ± 1.03, *Wrightia tinctoria* 5.90 ± 1.55, *Casuarina equisetifolia* 5.36 ± 0.46, *Polyalthia longifolia* 5.30 ± 0.34, *Tamarindus indica* 5.26 ± 1.09, *Eucalyptus tereticornis* 5.10 ± 1.03.

Moderate level of ion chelating activity was observed with *Millettia pinnatta* 4.16 ± 0.92, *Cicca acida* 4.00 ± 0.17, *Tectona grandis* 3.83 ± 0.80, *Annona squamosa* 3.43 ± 0.46, *Ficus benghalensis* 3.40 ± 0.34, *Azadirachta indica* 3.06 ± 0.11, *Ficus religiosa* 3.03 ± 0.11.

Similar result was reported by Krishnaveni for *Annona squamosa, Psidium guajava,*[11] *Tamarindus indica,*[15] *Ficus religiosa*[15,16] *Polyalthia longifolia,*[17] *Ficus benghalensis.*[17]

Hydrogen Peroxide Activity

Hydrogen peroxide activity was high with *Ficus religiosa* 4.27 ± 0.39, *Annona squamosa* 4.24 ± 0.46. Moderate with *Ficus benghalensis* 3.74 ± 0.03, *Eucalyptus tereticornis* 3.73 ± 0.05, *Santalum album* 3.73 ± 0.28, *Cicca acida* 3.30 ± 0.78, *Polyalthia longifolia* 3.25 ± 0.60, *Casuarina equisetifolia* 3.16 ± 0.40, *Tamarindus indica* 2.70 ± 0.00, *Artocarpus heterophyllus* 2.63 ± 0.11, *Wrightia tinctoria* 2.62 ± 0.47, *Tectona grandis* 2.60 ± 1.39, *Psidium guajava* 2.50 ± 0.43, *Azadirachta indica* 2.46 ± 0.40.

Low level of hydrogen peroxide activity was observed with *Millettia pinnatta* 1.66 ± 1.78.

Total Phenolics

Annona squamosa 7.43 ± 1.50, showed very high level of total phenolics followed by *Wrightia tinctoria* 6.70 ± 0.69, *Azadirachta indica* 6.16 ± 1.78, *Millettia pinnatta* 5.43 ± 2.54, *Artocarpus heterophyllus* 5.33 ± 1.78, *Casuarina equisetifolia* 5.23 ± 2.71, *Santalum album* 4.96 ± 0.75, *Polyalthia longifolia* 4.76 ± 0.92, *Ficus benghalensis* 4.70 ± 2.07, *Cicca acida* 4.13 ± 0.80, *Tamarindus indica* 4.10 ± 1.55, *Psidium guajava* 3.50 ± 0.69.

Very low level was observed with *Ficus religiosa* 2.00 ± 0.17 and *Tectona grandis* 1.73 ± 0.11.

Similar result was reported by Krishnaveni for *phenolics* with respect to *Ficus benghalensis,*[12] *Tectona grandis,*[12] *Tamarindus indica,*[12] *Artocarpus heterophyllus,*[14] *Casuarina equisetifolia,*[14,15] *Psidium guajava,*[15] *Polyalthia longifolia*[17,18] *Ficus religiosa.*[17]

Table 1: Antioxidant activity of plant leaves collected near Indian oil Gas plant

S. No	Name of the plants	Total antioxidant assay (mg/g)	Reducing power assay (mg/g)	Nitric oxide scavenging activity (mg/g)	Metal chelating activity(mg/g)	Hydrogen peroxide scavenging activity (%)
1.	*Tectona grandis*	1.13 ± 0.11	2.93 ± 0.02	2.73 ± 0.28	3.83 ± 0.80	2.60 ± 1.39
2.	*Ficus religiosa*	2.53 ± 0.63	1.73 ± 0.37	4.30 ± 0.00	3.03 ± 0.11	4.27 ± 0.39
3.	*Millettia pinnatta*	4.03 ± 1.50	2.93 ± 1.58	3.98 ± 1.15	4.16 ± 0.92	1.66 ± 1.78
4.	*Psidium guajava*	2.60 ± 0.51	2.71 ± 0.58	2.97 ± 1.52	6.10 ± 1.03	2.50 ± 0.43
5.	*Eucalyptus tereticornis*	3.40 ± 0.51	2.15 ± 0.34	3.68 ± 1.41	5.10 ± 1.03	3.73 ± 0.05
6.	*Tamarindus indica*	3.00 ± 0.69	3.90 ± 0.30	3.27 ± 1.78	5.26 ± 1.09	2.70 ± 0.00
7.	*Azadirachta indica*	3.83 ± 1.15	1.30 ± 0.00	3.41 ± 0.14	3.06 ± 0.11	2.46 ± 0.40
8.	*Santalum album*	3.16 ± 0.92	2.86 ± 0.14	3.46 ± 1.01	6.83 ± 1.67	3.73 ± 0.28
9.	*Casuarina equisetifolia*	5.00 ± 0.86	2.85 ± 0.25	4.38 ± 1.50	5.36 ± 0.46	3.16 ± 0.40
10.	*Annona squamosa*	4.63 ± 1.15	3.23 ± 0.80	2.20 ± 0.77	3.43 ± 0.46	4.24 ± 0.46
11.	*Cicca acida*	4.86 ± 0.75	3.20 ± 1.03	5.13 ± 0.11	4.00 ± 0.17	3.30 ± 0.78
12.	*Wrightia tinctoria*	3.20 ± 0.69	2.18 ± 0.02	3.67 ± 1.87	5.90 ± 1.55	2.62 ± 0.47
13.	*Artocarpus heterophyllus*	4.13 ± 0.11	4.20 ± 0.69	2.17 ± 0.56	6.13 ± 2.54	2.63 ± 0.11
14	*Polyalthia longifolia*	4.47 ± 0.63	3.18 ± 0.20	2.14 ± 0.53	5.30 ± 0.34	3.25 ± 0.60
15	*Ficus benghalensis*	5.10 ± 0.17	3.26 ± 0.92	3.51 ± 0.92	3.40 ± 0.34	3.74 ± 0.03

Values are Mean ± SD for three experiments

Table 2: Secondary Metabolites

S. No	Name of the plants	Total phenolics (mg/g)	Total Flavonoids (mg/g)
1.	*Tectona grandis*	1.73 ± 0.11	5.73 ± 0.28
2.	*Ficus religiosa*	2.00 ± 0.17	5.30 ± 0.86
3.	*Millettia pinnatta*	5.43 ± 2.54	2.83 ± 1.15
4.	*Psidium guajava*	3.50 ± 0.69	6.20 ± 0.69
5.	*Eucalyptus tereticornis*	2.60 ± 0.34	6.36 ± 0.23
6.	*Tamarindus indica*	4.10 ± 1.55	3.30 ± 1.03
7.	*Azadirachta indica*	6.16 ± 1.78	4.50 ± 0.17
8.	*Santalum album*	4.96 ± 0.75	4.73 ± 0.63
9.	*Casuarina equisetifolia*	5.23 ± 2.71	5.46 ± 1.96
10.	*Annona squamosa*	7.43 ± 1.50	5.33 ± 1.84
11.	*Cicca acida*	4.13 ± 0.80	4.23 ± 0.63
12.	*Wrightia tinctoria*	6.70 ± 0.69	2.18 ± 0.02
13.	*Artocarpus heterophyllus*	5.33 ± 1.78	4.20 ± 0.69
14	*Polyalthia longifolia*	4.76 ± 0.92	3.18 ± 0.20
15	*Ficus benghalensis*	4.70 ± 2.07	3.26 ± 0.92

Values are Mean ± SD for three experiments

Total Flavonoids

The flavonoid content was high in *Eucalyptus tereticornis* 6.36 ± 0.23, *Psidium guajava* 6.20 ± 0.69, *Tectona grandis* 5.73 ± 0.28, *Casuarina equisetifolia* 5.46 ± 1.96, *Annona squamosa* 5.33 ± 1.84, *Ficus religiosa* 5.30 ± 0.86. Moderate amount was observed with *Santalum album* 4.73 ± 0.63, *Azadirachta indica* 4.50 ± 0.17, *Cicca acida* 4.23 ± 0.63, *Artocarpus heterophyllus* 4.20 ± 0.69, *Tamarindus indica* 3.30 ± 1.03, *Ficus benghalensis* 3.26 ± 0.92, *Polyalthia longifolia* 3.18 ± 0.20, *Millettia pinnatta* 2.83 ± 1.15. Similar result was reported by Krishnaveni for *Tamarindus indica,*[10,13] *Ficus religiosa,*[13] *Polyalthia longifolia,*[16] with respect to flavonoids. Life on earth require oxygen for its existence.[19] So, it is essential to protect the plants so as to utilize it for the benefit of human being.

CONCLUSION

The total antioxidant activity was predominant in *Ficus benghalensis*. While, *Artocarpus heterophyllus* for reducing power activity, *Cicca acida* for nitric oxide scavenging activity, *Santalum album* for metal chelating activity, *Ficus religiosa* for hydrogen peroxide scavenging activity. The secondary metabolite phenolics was high with *Wrightia tinctoria* and *Eucalyptus tereticornis* showed highest flavonoid content. These activities confirm it to be a good antioxidant agent.

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REFERENCES

1. Prieto P, Pineda M, Agulia M, Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of Vitamin E, Analytical Biochemistry, 269, 1999, 337-341.
2. Oyaizu M, Studies on products of browning reaction prepared from glucosamine, Japan Journal of Nutrition, 44, 1986, 307-315.
3. Garrat DC, The quantitative analysis of drugs, Champman and Hall, Japan, 3,1964, 456-458.
4. Sreena KP, Poongothai A, Soundariya SV, Srirekha G, Santhi R, Annapoorani S, Evaluation of *in vitro* free radical scavenging efficacy of different organic extracts of Morinda tinctoria leaves, Int.J Pharm Sci, 3(3), 2011, 207-209.
5. Decker EA, Welch B, Role of ferritin as a lipid oxidation catalyst in muscle food, J of Agri. and Food Chem., 38, 1990, 674-677.
6. Nabavi SM, Ebrahimzadeh MA, Nabavi SF and Jafari M, Free radical scavenging activity and antioxidant capacity of *Eryngium caucasicum Trautv* and *Froripia subpinnata*. *Pharmacologyonline,* 3, 2008b, 19-25
7. Makkar HPS, Bluemmel M, Borowy NK, Becker K, Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods, J Sci Food Agr, 61, 1993, 161-165.
8. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Hamidinia A and Bekhradnia AR, Determination of antioxidant activity, phenol and flavonoids content of *Parrotia persica Mey.* Pharmacologyonline, 2, 2008a, 560-567.
9. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Fazelian M and Eslami B, *In vitro* antioxidant and free radical scavenging activity of *Diospyros lotus* and *Pyrus boissieriana* growing in Iran, *Pharmacognosy Magazine,* 4(18), 2009a, 123-127.
10. Krishnaveni M, Madhaiyan P, Durairaj S, Amsavalli L, Chandrasekar R, Antioxidant activity of plants at Chinnathirupathi, Salem, Tamil Nadu, India, International Journal of Pharmaceutical Sciences and Research, Society of Pharmaceutical Sciences and Research, 4(10), 2013, 3917-3919.
11. Krishnaveni M, Chandrasekar R, Amsavalli L, Durairaj S, Madhaiyan P, Free radical scavenging activity of plants at Perumalmalai Hill, International Journal of Pharmaceutical Sciences Review and Research, 21(1), 2013, 155-159.
12. Krishnaveni M, Amsavalli L, Chandrasekar R, Madhaiyan P, Durairaj S, Antioxidant activity of plants at Govt. College of Engineering Campus, Salem, TamilNadu, India, International Journal of Pharmaceutical Sciences Review and Research, 21(1), 2013,160-163.
13. M Krishnaveni and Jasbin Shyni George, Comparative study on APTI, antioxidant status of plants & soil health, Lambert academic publishing, ISBN: 978-3-659-52656-5, 2014, 1-64.
14. Krishnaveni M, Kalimuthu R, Ponraj K, Lavanya K, Magesh P, Jasbin shyni G, Antioxidant activities of plants studied in yercaud road sides, Salem, Tamil Nadu, India. International Journal of Pharmaceutical Sciences Review and Research, 27(1), 2014, 61-65.

15. Krishnaveni M, Ponraj K, Kalimuthu R, Lavanya K, Mahesh P, Jasbin Shyni G, Antioxidant activity of plants studied at Thoppur hill road sides, Dharmapuri, Tamil nadu, India. International Journal of Pharmaceutical Sciences Review and Research, 26(2), 2014, 171-176.

16. Krishnaveni M, Mahesh P, Ponraj K, Kalimuthu R, Lavanya K, Jasbin Shyni G, A comparative study on antioxidant activities of selected plants from road side, Salem, Tamil Nadu, India, International Journal of Pharmaceutical Sciences Review and Research, 26(2), 2014, 112-116.

17. Krishnaveni M, Kaveri L, Magesh P, Ponraj K, Kalimuthu R and George Jasbin Shyni, Free radical scavenging activity of selected plants, World journal of pharmacy and pharmaceutical sciences, 3(5), 2014, 765-775.

18. Krishnaveni M, Durairaj S, Madhiyan P, Amsavalli L and Chandrasekar R, Invitro Free Radical Scavenging activity of aqueous leaf extract of plants near thermal power plant, Mettur, Salem, International Journal of Pharmaceutical Sciences and Research, 4(9), 2013, 3659-3662.

19. Krishnaveni M, Antioxidant activities of selected plants, International Journal of Pharmacy and Pharmaceutical Sciences, 6(2), 2014, 126-128.

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