Biological Forum – An International Journal **15(2): 1101-1106(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Estimation of Total Phenolic and Flavonoid Content and Antioxidant Activity in *Catharanthus roseus* L. (Alba) Plant Extract

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(Received: 01 January 2023; Revised: 18 February 2023; Accepted: 20 February 2023; Published: 23 February 2023)
(Published by Research Trend)

ABSTRACT: The Phenolic compounds are important plant metabolite which have redox properties liable for antioxidant activity. The hydroxyl group of phenolic compounds facilitate free radical scavenging. The Total Phenolic Content of each extract of leaf, stem, root, and flower were determined by the Folin-Ciocalteau method and calculated as Tannic acid equivalents (TAE) g^{-1}. This has also been used to compute antioxidants in multifaceted biological structures. DPPH method is being used to measure the antioxidant activity of methanolic extract. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and gives purple color. These leaf extract have maximum phenolic content 1378 µg and for Flower extract it is 1185 µg from regression curve. The Total flavonoids were found in leaf and Flower extract are 0.60% and 1.70% respectively. The antioxidant activity of leaf extract was derived from a calibration curve (Y=0.2934x + 40.605, R^2 = 0.9079) and for flower extract (Y=12.982x – 415.8, R^2 = 0.9365) from this calibration curve. So R^2 value is maximum for Leaf and Flower extract. The IC_{50} value of stem sample is 688.47µg/ml therefore inhibition activity or antiradical efficiency is maximum of stem extract of *Catharanthus roseus* L. (Alba). By comparing the correlation coefficients (R-values), it could be suggested that phenolic and flavonoid groups are highly accountable for the antioxidant activity. This study can also prove by comparing antidiabetic drug metformin and nifedipine, the aqueous extract of the leaves of *Catharanthus roseus* by conducting experiment on sugar induced wistar rats, *Catharanthus roseus* leaves extract demonstrate better antioxidant activity in the treatment of diabetes and hypertension.

Keywords: Antioxidant activity, Total phenolic content, Regression curve, IC_{50} value, Antiradical efficiency, 2, 2-diphenyl- 1-picrylhydrazyl (DPPH), R^2.

INTRODUCTION

Phenols, also known as phenolics, are a type of chemical compounds that consist of an aromatic hydroxyl group (-OH) than are directly bounded together. Carbolic acid C_6H_5OH is the simplest class of phenol. Phenolics compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule (Amorati and Valgimigli 2012; Khoddami *et al*., 2013; Robbins, 2003).

The hydroxyl group can be free or attached in another function as ether, ester or glycoside (Bruneton, 1999). They are widely distributed in plants and particularly present either as soluble or cell wall bound compounds, because of interaction of a plant with its environment (Matern *et al*., 1995). This incorporates C_6C_1 compounds few of them named as 2,3-dihydroxybenzoic acid and phenylpropanoids such as cinnamic acid derivatives, anthocyanins and flavonoids. Organisms that synthesize phenolic compounds do so in response to ecological pressures such as pathogen and insect attack, UV radiation and wounding (Klepacka, 2011).

Phenolics hinder oxidative degradation of lipids and thereby raise the excellence and nutritional value of food. Phenolics possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic as well as ability of modifying gene expression (Mishra and Tiwari 2011). Food consumed by human in diets including various forms of plants being used in consumption by human, traditional medicine of various cultures, their role in human health and disease is a subject of research (Laldingliani *et al*., 2022; Kumar *et al.,* 2022; Amorati and Valgimigli 2012; Klepacka, 2011; Mishra and Tiwari 2011).

Flavonoids are polyphenolic molecules covering 15 carbon atoms and soluble in water. Some of the best-known flavonoids including quercetin, kaempferol, rutin, catechins and anthocyanidins. They provide health benefits through cell signalling pathways and antioxidant effects. Flavonoids are a group of plant metabolites which have known properties like free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Frankel *et al*., 1995). The water extract of the plant showed higher antioxidant potential than the acetone and DMSO extracts of the plant (Mir *et al*., 2018). Due to its antioxidant properties, it can interfere with the oxidative process by reacting with free radicals chelating, catalytic

metals and by acting as oxygen scavengers (Dreosti, 2002). Free radicals play of the essence role in the development of tissue damage in various human diseases such as cancer, aging, neurodegenerative, arteriosclerosis, antiviral and pathological events in living organism (Erdemoglu *et al*., 2006; Gutteridge, 1994). According to Pham *et al*. (2018) the *n*-butanol fraction of *Catharanthus roseus* contained the highest levels of saponins and phenolics (3037.54 mg ESE/g and 77.87 mg GAE/g, respectively) and possessed the strongest antioxidant capacity amongst the tested extracts. HPLC analysis also revealed that this *n*-butanol fraction had high levels of apigenin and kaempferol. Antioxidants may have an important in the prevention of these diseases. Natural antioxidants or phytochemical antioxidants are the secondary metabolites of plants (Walton& Brown 1999). Antioxidants are the substance that when present in low concentrations compared to oxidisable substrate, they delay or prevents the oxidation of that substance (Halliwell and Gutteridge 1989).

MATERIAL AND METHODS

The Experiments for extraction and estimation of antioxidants were conducted in phase by phase. In first phase Extraction were carried out and then estimation of antioxidants was initiated and concluded.

1. Extraction of Total Phenolic Content
2. Extraction of Flavonoids
3. DPPH radical scavenging method
4. Procedure and Formula

1. Extraction of Total Phenolic Content: "Folin-Ciocalteau" method is being used to determine the extraction of total phenolic content. 2g sample in 25 ml methanol kept for incubation till 48 hours was filtered with Whatman no.1 paper. 0.5 ml of the sample was added to 5 ml Folin reagent (2 ml Folin + 18 ml distilled water) of 0.2 N Folin-Ciocalteau reagent (1:10) and placed for 5 minutes. 4 ml of Na_2CO_3 were then added and the mixture was allowed to stand for 15 minutes. Absorbance was measured at 765 nm against the blank (Methanol) using 1 cm cuvette in (UV/VIS-5300) spectrophotometer. Tannic acid (0 - 1000 mg l^{-1}) was used to produce standard calibration curve. Tannic acid as a natural antioxidant used for comparison. The total phenolic content was expressed in µg of Tannic acid equivalents (TAE) g^{-1}.

2. Extraction of Flavonoids: Different plant parts (leaves, stems, roots, and flowers) were air dried and powdered, separately.20 gm powdered of each sample extracted separately with 80% methanol on water bath for 24hrs (Subramanian and Nagarajan 1969). The methanol soluble fractions were filtered, concentrated *in vacuo* and aqueous fractions were fractioned by sequential extraction with petroleum ether (Fraction I), diethyl ether (Fraction II) and ethyl acetate (Fraction III) separately. Each step was repeated thrice for complete extraction, fraction I (Fr I) was discarded in each case because it contained fatty substance, whereas fraction II (Fr II) and fraction III (Fr III) free flavonoids were concentrated were being used for determining flavonoids. After drying Fr III was further hydrolyzed by refluxing with 7% sulphuric acid for making bound flavonoids (10mLg^{-1} per plant material for 2h), filtered and filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled separately, neutralized by distilled water with repeated washings and concentrated *in vacuo* and bound flavonoids of Fr III were determined by weighing machine.

3. DPPH radical scavenging method: 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) is being used to evaluate antioxidant activity in complex biological systems in recent times. The DPPH method can be used for solid or liquid samples, and it is not specific to any antioxidant component but applies to the overall antioxidant capacity of the sample. A strong absorption observed in free radical of DPPH, maximum at 517 nm and gives purple color. The color turns from purple to yellow as the molar absorptive of the DPPH radical at 517 nm, when the odd electron of DPPH radical becomes paired with hydrogen. To reduced DPPH from a free radical scavenging antioxidant is being paired with hydrogen. The outcome of decolorization is with respect to number of electrons captured. So, antioxidant activity is expressed as % discoloration of 2,2-diphenyl-1-picrylhydrazyl Antioxidant compounds may be water-soluble, lipid-soluble, insoluble, or bound to cell walls. Therefore, extraction efficiency is a crucial factor in determination of antioxidant activity. Antioxidant activity has specified in many ways including the percentage of the reagent used, the oxidation inhibition rate and so on. The chemical reaction occurrence of DPPH in plant shown in Fig. 1. An easier way to present antioxidant activity of foods would be a common source for a reference standard. Common reference standard like Ascorbic Acid (Vitamin A, C and E) serves this purpose (Cavin *et al*., 1998; Kirby and Schmidt 1997; Brand-William *et al*., 1995).

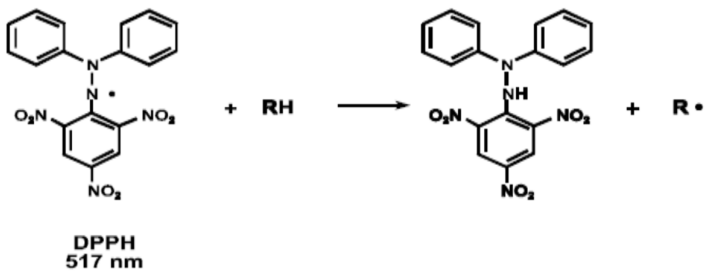

Fig. 1. Chemical reaction occurrence of DPPH in plant samples, which is used to evaluate antioxidant activity.

4. Procedure and formula used:

Procedure: The different extracts were measured in terms of hydrogen donating or radical scavenging ability using the stable radical $DPPH^{\cdot}$ (Brand-Williams *et al*, 1995). 1ml of a methanolic solution of the extract at different concentrations ranging from 1 to 1000 µg ml^{-1} was mixed with 1 ml of a DPPH methanolic solution (0.01gm DPPH in 100 ml methanol). The absorbance measured at 517 nm after 20 min of reaction. The methanol mixes with DPPH used as a blank. The DPPH percentage discoloration of the sample was determined according to the formula.

Formula: % Decoloration = [1 − (Abs SAMPLE/Abs CONTROL)] × 100

The decoloration plotted between the sample extract concentration and % inhibition, a logarithmic regression curve was established to calculate the IC_{50} value. The IC_{50} value is Half-maximal inhibitory concentration, most widely used and measure the drug's efficacy. Antioxidant substance (amount of sample)) required to scavenge 50% of the initial DPPH radicle. The lesser the IC_{50} value, the substance at scavenging DPPH is more effective and this implies a higher antioxidant activity.

RESULTS AND DISCUSSION

The leaf extract has maximum phenolic content 1378 µg and for Flower extract is 1185 µg and Total flavonoids were found in leaf and Flower extract is 0.60% and 1.70% from regression curve as respectively as shown in Table 1. The antioxidant activity of leaf extract derived from a calibration curve (Y=0.2934x + 40.605, R^2 = 0.9079) and for flower extract (Y=12.982x – 415.8, R^2 = 0.9365) as shown in Table 2. So R^2 value is maximum for Leaf and Flower extract. The IC_{50} value of stem sample is 688.47µg/ml therefore inhibition activity or antiradical efficiency is maximum of stem extract of *Catharanthus roseus* L.(Alba). By comparing the correlation coefficients (R-values) we can say that phenolic and flavonoid groups are highly responsible for the antioxidant activity (Aryal *et al*., 2019). The antioxidants act either by scavenging various types of free radicals derived from oxidative process, by preventing free radical formation through reduction precursors or by chelating metals (Bors *et al*., 1984; Burton and In gold 1984; Mark *et al*., 1994). The reduction of DPPH assay has been used to detect products with antioxidants activity as free radical scavengers (Cavin *et al*., 1998; Gamez *et al*., 1998; Tubaro *et al*., 1996). In this study all the extract were shown to possess significant DPPH radical scavenging activity. To identified the IC_{50} value of different plant parts standard calibration curve are plotted between % inhibition and samples amount as shown in Fig. 2-5. It has been reported that *Catharanthus roseus* L. leaf extract contains relatively large quantity of Phenolic (antioxidant) compounds is 1378 µg as shown in Table 1 and Fig. 2 and but the IC_{50} value is less of stem sample is 688.47µg/ml as shown in Table 2, so inhibition activity or antiradical efficiency is maximum of stem extract of *Catharanthus roseus* L. This view is also supported by Rasool *et al.,* (2011). But according to Jain *et al.* (2014), maximum number of phenolic contents were found in stem (Jain *et al*., 2014). Leaf extract showed maximum superoxide radical scavenging activity than other plant parts while root extract have maximum 2,2- diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity.As per wistarrats 60% fructose-induced with aqueous extract of *Catharanthus roseus* leaves increased serum levels of catalase, superoxide dismutase, malondialdehyde, kidney and heart, and oxide nitric in the aorta. This surge was studied at doses ranging from 200 to 1000 mg/kg. Compared to nifedipine and metformin, the aqueous extract of the leaves of *Catharanthus roseus* appears to have antioxidant activity imply in the treatment of hypertension induced by fructose. As per Nisar *et al.* (2017); Thanga Revathi *et al*. (2018) maximum antioxidant activity was found in flower petals extract. The leaves of *C. roseus* showed good antioxidant activity (Akbar 2020; Kaur and Mondal 2014; Patharajan *et al*., 2014; Tiong *et al*., 2013). According to Biswas *et al.* (2010), highest DPPH radical scavenging activity was set up in *Catharanthus roseus* L. (white variety). Ferreres *et al*. (2008) reported the scavenging capability for the waterless leaves extract of this plant against DPPH (IC50 at 447 µg/mL), superoxide and nitric oxide particles. Zheng and Wang (2001) have revealed the extract of this plant has the highest ORAC antioxidant capacity out of several medicinal herbs screened, with ORAC values of 22.30 µmol Trolox equivalent (TE)/g of fresh weight. The antioxidant activity of this plant was usually retrieved along with their phenolic content as it demonstrated high direct correlation with activity. So, there is linear correlation between Total Flavonoids, Total phenolic content, and antioxidant activity. In this study, antioxidant activity indicates extract of leaf and flower sample with higher phenolic and flavonoid contents could be a significant source of natural antioxidants But the IC_{50} value is less of stem sample. The lower the IC_{50} value, the more potent is the substance at scavenging DPPH and that indicate higher antioxidant activity. As per O New Lee *et al.* (2020), total of 59, 55, and 39 compounds were noticed in the callus, somaclonal variant shoot, and normal green shoot tissues, respectively. The normal green shoot isolates shown the best free radical scavenging capability and reducing power activity. The IC_{50} value is Half-maximal inhibitory concentration, most widely used and measure the drug's efficacy. Plant parts from which the phytochemicals were isolated or extracts was prepared botanically-derived antibiotics as a template for antimicrobial drug development (Uttpal *et al*., 2020). So, parts of *Catharanthus roseus* that grow above the ground used to make medicine.

Table 1: Estimation of total Phenolic content & Flavonoids in plant parts of *Catharanthus roseus* L. (Alba) through regression curve.

Sr. No.	Plant Part	Total phenolic content (in µg)	FrIII (Free Flavonoids)	FrIII (Bound Flavonoids)	Total Flavonoids (Percentage)
1.	Leaf	1378	0.30	0.30	0.60
2.	Stem	1159	0.25	0.25	0.50
3.	Root	872	0.15	0.10	0.25
4.	Flower	1185	1.45	0.25	1.70

Table 2: Calculation of IC_{50} values for determination of highest antioxidant activity in plant parts of *Catharanthus roseus* L. (Alba).

Sr. No.	Plant Part	Regression equation	R2	X	IC50 (µg ml-1)
1.	Leaf	Y=0.2934x+40.605	0.9079	32.0211	3202.00
2.	Stem	Y=8.812x-10.668	0.8679	6.8847	688.47
3.	Root	Y=0.5347x + 8.302	0.8210	77.9839	7798.39
4.	Flower	Y=12.982x -415.8	0.9365	35.8806	3588.00

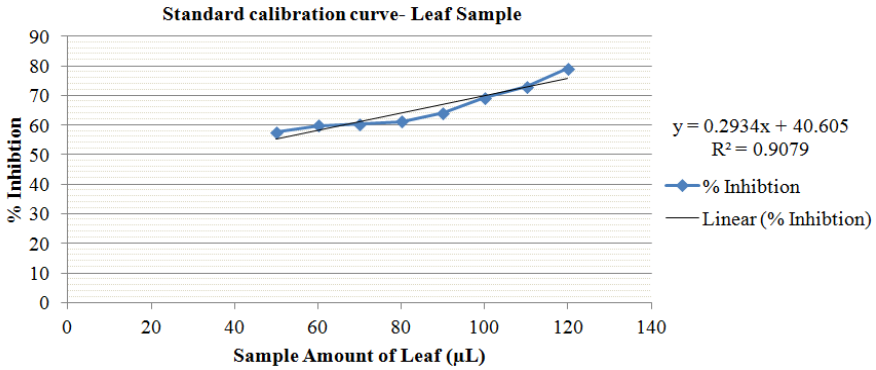

Fig. 2. Standard calibration curve of leaf part and inhibit response to identify IC_{50}.

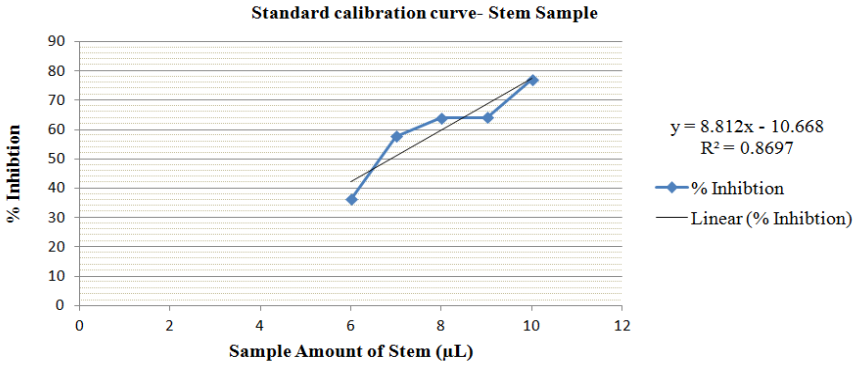

Fig. 3. Standard calibration curve of stem part and inhibit response to identify IC_{50}.

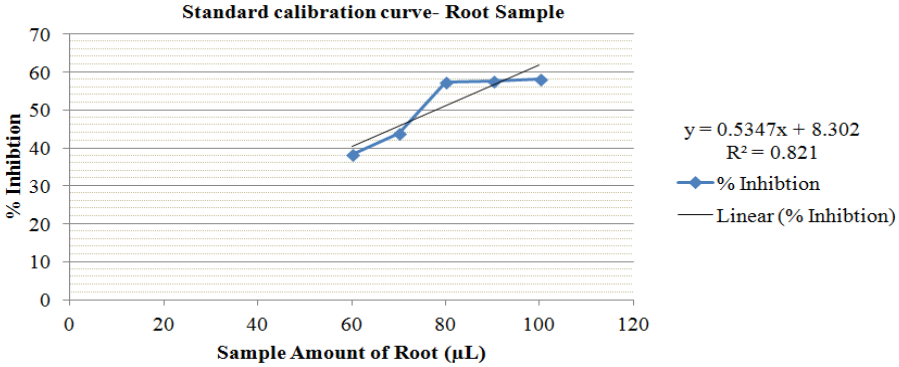

Fig. 4. Standard calibration curve of Root part and inhibit response to identify IC_{50}.

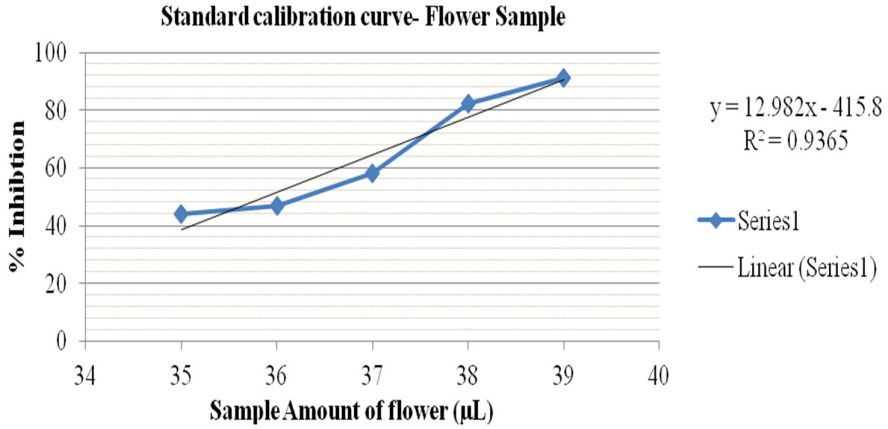

Fig. 5. Standard calibration curve of Flower part and inhibit response to identify IC_{50}.

CONCLUSIONS

The phenolic and flavonoid groups are highly responsible for the antioxidant activity. So, there is linear correlation between Total Flavonoids, Total phenolic content, and antioxidant activity. In this study, antioxidant activity indicates extract of leaf and flower sample with higher phenolic and flavonoid contents could be a significant source of natural antioxidants. But the IC_{50} value is less of stem sample. The lower the IC_{50} value, the more potent is the substance at scavenging DPPH and this indicate higher antioxidant activity.

FUTURE SCOPE

This study can be tapped commercially to increase the synthesis of the medicinal drugs due to Antioxidant properties.

Author contributions. Priyanka Tolambiya collected materials, designed, performed the experiments, and analysed the data. All authors contributed to the writing and revising the manuscript. All authors have read and approved the final manuscript.

Acknowledgement. The Authors are thankful to University of Rajasthan, Jaipur for providing sophisticated lab facility and relevant literature to conduct the experiment.

Conflict of Interest. None.

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How to cite this article: Tolambiya Priyanka, Jagetiya B.L., Mathur Sujata (2023). Estimation of Total Phenolic and Flavonoid Content and Antioxidant Activity in *Catharanthus roseus* L. (Alba) Plant Extract. *Biological Forum – An International Journal, 15*(2): 1101-1106.