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Somnath Kangralkar
Department of Botany, Ramniranjan Jhunjhunwala College, Mumbai, Maharashtra, India

Anil Avhad
Department of Botany, Ramniranjan Jhunjhunwala College, Mumbai, Maharashtra, India

Corresponding Author:
Somnath Kangralkar
Department of Botany, Ramniranjan Jhunjhunwala College, Mumbai, Maharashtra, India

Unveiling the antioxidant power: *In-vitro* analysis of different plant parts of *Wrightia tinctoria* R. Br.

Somnath Kangralkar and Anil Avhad

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Abstract
Oxidative stress, caused by an imbalance between reactive oxygen species (ROS) and antioxidant defences, is a major contributor to cellular damage and various degenerative diseases. In this study, the antioxidant activity and bioactive compound content of different parts of *Wrightia tinctoria* were evaluated using chemical assays. Total polyphenol content (TPC) and total flavonoid content (TFC) were determined using the Folin-Ciocalteu and $AlCl_3$ spectrophotometric methods, respectively. Antioxidant activity was assessed through DPPH radical scavenging and Phosphomolybdenum assays. The results showed that leaves exhibited the highest TPC (4072 mg GAE/100g) and TFC (379 mg QE/100g), followed by fruits, stems, and bark. DPPH assay revealed strong radical scavenging activity, with the lowest IC_{50} value observed in leaves (1.08 mg/ml), indicating the highest antioxidant potential. The Phosphomolybdenum assay further supported these findings, demonstrating significant reducing capacity in the tested plant parts. A strong correlation was observed between total phenolic and flavonoid content with antioxidant activity (R^2 = 0.9995 for TFC and R^2 = 0.9791 for TPC), highlighting their crucial role in mitigating oxidative stress. These findings suggest that *Wrightia tinctoria*. Possesses potent natural antioxidants, making it a promising source for nutraceutical and pharmaceutical applications**.**

Keywords: Oxidative stress, antioxidant activity, bioactive compounds, radical scavenging activity, reducing capacity, phenolic compounds

1. Introduction

1.1 Oxidative stress

Oxidative stress was first characterized by Sies (1985, 1986) as a significant disruption in the equilibrium between oxidants and antioxidants, described as "a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage." While this definition appears straightforward, it is predicated on more complex concepts related to oxidation, antioxidants, and the notion of balance. The concept of oxidation itself is seemingly simple: it involves the loss of electrons by a species, the gain of oxygen, or the loss of hydrogen. However, the process of oxidation inherently requires a corresponding reduction of another species. The implications of this process are context dependent. Buettner (1993) elaborates on this by introducing a hierarchy of oxidants. In biological systems, substances that rank high on this hierarchy, such as the hydroxyl (OH^-) radical, are almost invariably oxidants. Conversely, other substances, like nitric oxide ($NO\cdot$) or hydrogen peroxide (H_2O_2), can function as either oxidants or reductants, contingent upon their interactions with other substances positioned higher or lower in the hierarchy. (Yoshikawa, 2002) [9].

1.2 Production of ROS

Reactive oxygen species (ROS) represent the active forms of oxygen, which are essential for various cellular functions both at rest and in response to diverse stimuli. The diatomic oxygen molecule (O_2) is characterized by its lack of spontaneous reactivity with other molecules due to the presence of two unpaired electrons, rendering it a biradical. For oxygen to engage in reactions with organic molecules, two pathways are available: the organic molecule can be converted into a monoradical by the removal of one electron (a process known as oxidation), and/or oxygen can be transformed into a monoradical through the addition of one electron (reduction). These reactions necessitate a significant energy input; thus, catalytic systems are essential to lower the energy barrier through a series of subsequent

low-energy steps. In biological systems, these catalytic systems typically consist of metallic complexes or enzymes, specifically oxygenases and oxidases, which facilitate the conversion of organic molecules or oxygen into monoradicals. Oxygenases, such as cyclooxygenases, catalyze the removal of a hydrogen atom (H^+) along with its electron from a substrate. Conversely, oxidases, like NADPH-oxidase, facilitate the transfer of an electron from a donor molecule to oxygen, resulting in the formation of superoxide (O_2). Superoxide can subsequently be converted into hydrogen peroxide (H_2O_2) through a process known as dismutation, which can occur spontaneously or enzymatically. Hydrogen peroxide is relatively stable, allowing it to diffuse away from its site of production. Potential subsequent reactions include detoxification into water via enzymes such as glutathione peroxidases or catalase, or through mitochondrial respiratory chain complexes,the generation of hydroxyl radicals (OH^-) in the presence of iron (e.g., ferrous ions in heme groups), or the conversion into hypochlorous acid (HOCl) by peroxidases. Hypochlorous acid acts as a potent oxidant, possessing a sufficient lifespan to diffuse across cellular membranes and oxidize a wide array of biomolecules. (Preiser, J. C. 2012) [4].

1.3 Bioactive antioxidants

Antioxidants are substances that, even in minimal amounts in food or the body, can slow down, control, or prevent oxidative processes that lead to a decline in food quality or the onset and progression of degenerative diseases in the body. Examples of antioxidants that fit this definition include agents that scavenge free radicals, quench singlet oxygen, inactive peroxides and other reactive oxygen species (ROS), chelates metal ions, neutralize secondary oxidation products, and inhibit pro-oxidative enzymes, among others. (Shahidi & Zhong, 2007) [8].

These compounds demonstrate their capacity to inhibit oxidation processes through a variety of mechanisms and differing levels of activity. They can be categorized based on their mode of action into primary and secondary antioxidants. Primary antioxidants, including tocopherols and certain phenolic compounds, disrupt the chain reaction of oxidation by functioning as hydrogen donors or free radical acceptors, leading to the formation of more stable radicals. This inhibitory reaction competes with the propagation phase of lipid oxidation, resulting in stable products that do not initiate new free radicals or trigger rapid oxidation through a chain reaction. (Nawar, 1996) [2].

The efficacy of antioxidants is typically affected by various factors, such as their structural characteristics, concentration levels, temperature, the nature of the oxidation substrate, the physical state of the system, and the presence of pro-oxidants and synergistic agents. (Yanishlieva-Maslarova, 2001) [7].

The purpose of the present study was to determine antioxidant activity, TFC and TPC by chemical assay in the The plant part considered for the study were Leaf, Stem, Bark, and Fruit of *Wrightia tinctoria* R. Br. Total polyphenol content was estimated by Folin-Ciocalteu method and Total flavonoids were quantified by the $AlCl_3$ method in terms of Quercetin equivalence. Total antioxidant capacity is measured using Phosphomolybdenum assay and Radical scavenging activity is determined using DPPH radical scavenging assay, which then compared with TPC and TFC to check the correlation between these two with antioxidant activity

1.4 Polyphenol determination using Folin-Ciocalteu reagent

The Folin-Ciocalteu method is based on an electron-transfer reaction, where antioxidants donate electrons, and the Folin-Ciocalteu reagent acts as the oxidizing agent, in an alkaline environment (pH 10, adjusted with sodium carbonate), phenolic compounds reduce phosphotungstic and phosphomolybdic acids, causing a colour change from yellow to blue. The intensity of this colour, measured at approximately 760 nm, is proportional to the reducing capacity of the phenolic compounds. The reaction involves reversible electron transfers, primarily reducing molybdenum (Mo^{6+} to Mo^{5+}), while tungstate is less readily reduced. The results are expressed as gallic acid equivalents (GAE). (Pérez, 2023) [10].

1.5 Flavonoids determination using Aluminium chloride

$AlCl_3$ method is based on formation of metal complex with flavonoids. The formation of a yellow-coloured complex between flavonoids and aluminium chloride is well-documented in scientific literature. Flavonoids with a 4-carbonyl group and hydroxyl groups at positions 3 or 5 form this complex, which can be measured spectrophotometrically at 415 nm

1.6 DPPH* radical scavenging activity

DPPH* (2, 2' -diphenyl-1-picrylhydrazyl radical), are stable synthetic radical which are purple in oxidised state which then turns into yellow after reduction by antioxidant molecule.

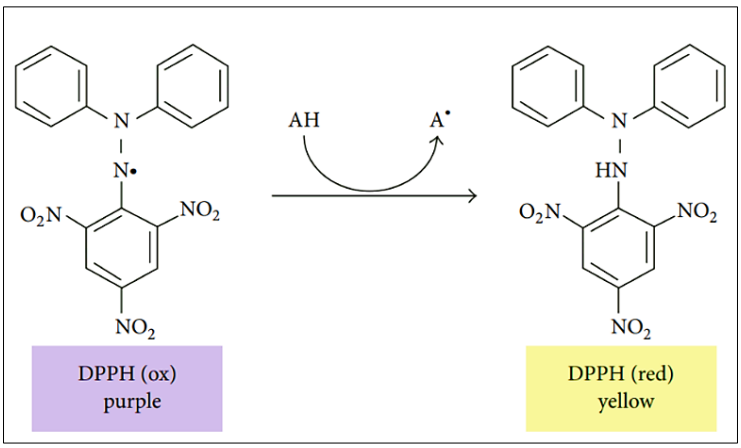

Fig 1: Principle of DPPH radical scavenging capacity assay. (Teixeira, 2013) [13].

1.7 Total antioxidant capacity (Phosphomolybdenum Assay)

The total antioxidant capacity assay can be used to determine the capacity of antioxidants through the formation of Phosphomolybdenum complex. The assay is based on the reduction of Mo (Vl) to Mo (V) by using sample analyte, which subsequently produces a green phosphate Mo (V) complex at acidic condition. (Phatak & Hendre 2014) [3].

2. Materials and Methods

2.1 Collection of Plant Materials

Plants materials were collected from their natural habitat, from Yeoor hills, Thane. The plant was identified and authenticated by Blatter herbarium, Mumbai

Plant was brought to the laboratory and washed using clean water to remove dust and pat dried before keeping separately as Leaf, Stem, Bark and Fruit in oven to completely dry at 60ºC, plant parts were processed into fine powder using Laboratory grinder and passed through 160-micron pore size sieve to obtain minimum and even particle size composition for further used for qualitative and quantitative estimations.

2.2 Plant extract preparation

Plant extracts were prepared using standardized method of assays, 1% extracts were prepared using 0.2g of plant part powder in 20 ml of solvent i.e. water, Methanol or ethanol for respective assay. The extract was prepared using ultrasonic bath sonicator for 30 min to ensure maximum extraction at room temperature, after extraction plant parts extracts were centrifuged at 2500 rpm for 5 mins to get clear supernatant which was further used for analysis.

2.3 Determination of total phenolics

Total phenolics were measured using Folin Ciocalteu reagent method, as described by Singleton and Rossi (1965) [12], with minor modification. Briefly, an aliquot 1 ml of appropriately diluted extracts or standard solutions of gallic acid (40, 80, 120, 160 and 200 mg/L) was added to a 25 mL volumetric flask containing 9 ml of DW. A reagent blank using DW was prepared. 1 ml of Folin Ciocalteu reagent was added to the mixture and vortex. After 5 min, 10 ml of 7% Na_2CO_3 solution was added with mixing. The solution was then consecutively diluted to volume 25 ml with DW and mixed thoroughly. After incubation for 90 min at 23°C, the absorbance versus prepared blank was read at 750 nm. Total phenolic content of leaf, stem, bark and fruit was expressed as mg gallic acid equivalents (GAE)/100 g dried sample. All samples were analysed in three replications.

2.4 Determination of total flavonoids

Total flavonoid content was determine using standard quercetin equivalence (Tristantini *et al*., 2019) [5]. Quercetin concentration is first estimated by Aluminium chloride spectrophotometric assay, 0.5ml of plant extract in 1.5 ml distilled water and 0.1 ml potassium acetate, 0.1 ml aluminium chloride is added subsequently, volume is raised to 5 ml by adding 2.8 ml of DW again. Quercetin 1mg/ml was used as a reference standard dilutions were made to achieve a calibration curve by standard series 5,10,15,20,25µg treating with the same method as a plant extract and absorbance was measured at 415nm keeping one test as blank without plant extract or quercetin, results are further analysed for estimation of quercetin.

Total Flavonoid content was calculated using equation as 1 mg of quercetin Equivalence (QE) per gram, and by multiplying quercetin concentration in ppm (C) by the volume of solution in ml (V) and dividing by mass of sample in grams (W) (Bhandari *et al*., 2014) [1].

2.5 DPPH* Radical scavenging assay

Total antioxidant activity was estimated by DPPH Radical scavenging assay, DPPH which is a stable radical was employed to measure the ability of different plant extracts to scavenge of to donate hydrogen. DPPH is a deep purple compound that turns pale yellow after reduction. Adding of plant extract to make respective series of plant parts with 3 ml DPPH (0.004% v/v in methanol), colour was measured after incubation at room temperature for 30 min in the dark (reduction in purple is directly proportional to the antioxidant activity). Gallic acid was used as a reference standard, same as the extracts, and absorbance is measured at 517 nm against methanol as control. Percentage inhibition activity is calculated using the formula (Tuanputra *et al* 2021) [6].

% Inhibition of DPPH* activity = (Ac - As / Ac) X 100

Where Ac = Absorbance of control, As = Absorbance of sample

Total antioxidant capacity (Phosphomolybdenum Assay)

Total antioxidant capacity was determined using a Phosphomolybdenum assay. (Untea A, *et. al*. 2018) [11] Plant extract (2% in D.W.)series was prepared by adjusting the volume to 1 ml and adding 1 ml phosphomolybdic reagent (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate).to make respective series of plant parts to see the trend of activity in increasing concentration of plant part extracts (Leaf, Stem, Bark Fruit) as shown in table no.1. Tubes were capped and kept for incubation for 60 mins at 95°C in hot water bath. After incubation samples were cooled at room temperature and absorbance is measured at 695 nm against a blank without standard or plant extract (1ml reagent + 1 ml distilled water) and Ascorbic acid (1 mg/ml) was used as a reference standard which reduce the phosphomolybdic reagent forming blue colour.

3. Results

3.1 Total Polyphenols Content

The significant quantity polyphenols were obtained in plant extracts of *Wrightia tinctoria* R. Br. of which leaf showed the highest Polyphenol content (4072mg GAE/100g), followed by the fruit (1681mg GAE /100g), the Bark (1624mg GAE /100g), and the Stem (1132mg GAE /100g). $y = 0.0041x - 0.0264$ $R^2 = 0.9995$

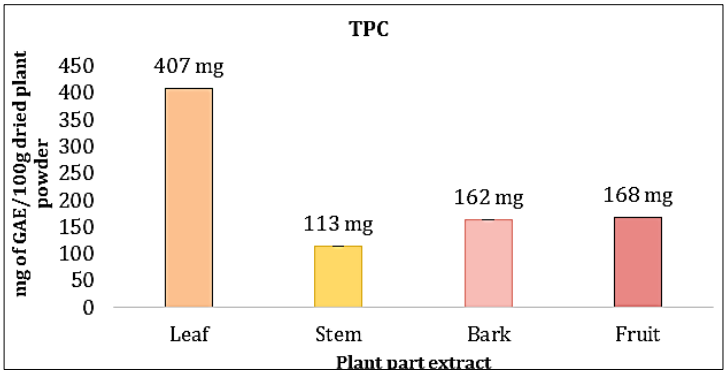

Fig 2: Total phenolic content in plant parts of *Wrightia tinctoria* R. Br.

3.2 Total Flavonoids Content

Flavonoids are abundantly found in the plant extracts of *Wrightia tinctoria* R. Br. Among these extracts, the leaves exhibited the highest concentration of Flavonoids, measuring (379 mg QE/g). Followed by the fruit, with (224 mg QE/g), stem with (113 mg QE/g), and bark with (24 mg QE/g). y = 0.0394x - 0.0087
R^2 = 0.9984

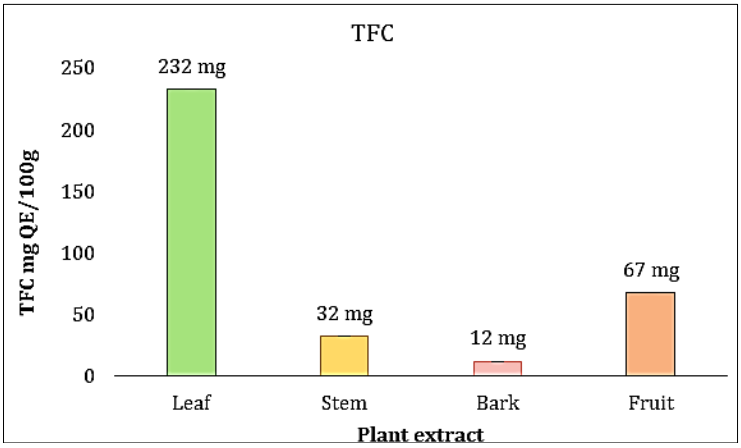

Fig 3: Total Flavonoid content in plant parts of *Wrightia tinctoria* R. Br.

3.3 DPPH* Radical Scavenging activity

DPPH solution shows a deep purple color and after complete reduction, it turns yellow. The reduction is measured by adding plant extracts, which reveal the potential of plant extracts to show antioxidant properties. DPPH activity of plant extracts is tested by gradually increasing the concentration to assess the antioxidant capacity and determine the effective concentration that achieves 50% inhibition (IC_{50}value) y =1.9721x-1.2095 R^2 = 0.9919

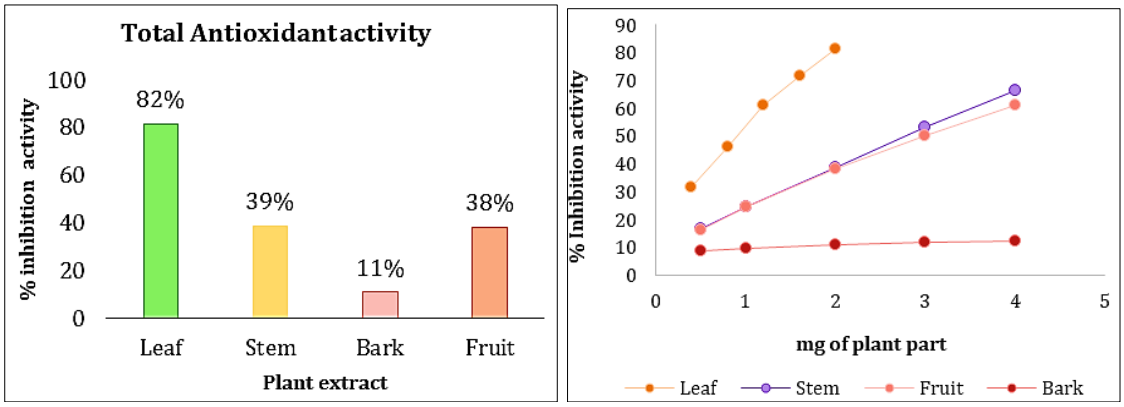

Fig 4: (a) Comparative antioxidant activity of 2mg of plant extracts (b) Percentage inhibition activity of different plant parts of *Wrightia tinctoria* R. Br

3.4 Phosphomolybdenum assay

Phosphomolybdenum assay is based on the reduction of Phosphate-Mo (VI) to Phosphate Mo (V) by the extract and formation of a bluish-green colored phosphate/Mo (V) complex in acid condition. Ascorbic acid is used as a positive control and absorbance is plotted against the concentration of ascorbic acid and also plant extracts were tested by gradually increasing the concentration to assess the capacity to determine the efficiency to show antioxidant activity (Pathak *et al* 2014) [3] y = 0.0328x - 0.0799 R^2 = 0.9998

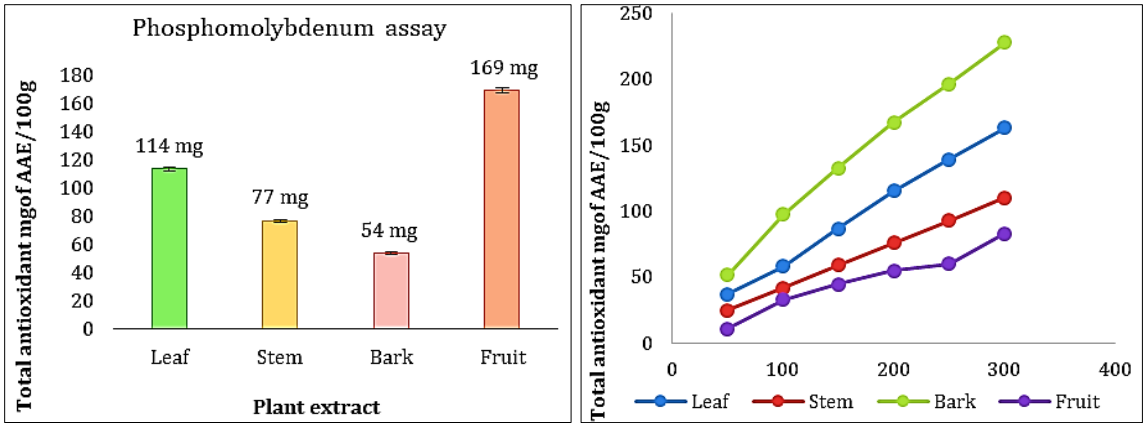

Fig 5: (a) Comparative Total antioxidant capacity (b) Antioxidant activity of different plant parts of *Wrightia tinctoria* R. Br.

3.5 Correlation antioxidant activity and bioactive compounds

Correlation between total phenolic content, total flavonoids and total antioxidant activity was determined by plotting IC_{50} (µg/ml) against total flavonoids content and total phenolic content. Direct correlation between DPPH radical scavenging activity and Total Phenolic content, total flavonoid content can be represented in linear regression. By correlating these parameters, it is revealed that radical scavenging activity and TPC, TFC are strongly correlated, with a coefficient of correlation R^2 = 0.9897. And the correlation coefficient between radical scavenging activity and TPC is R^2 = 0.9791 shows strong correlation (Bhandari *et al* 2014) [1].

Table 2: DPPH radical scavenging activity and TPC and TFC in different parts of *W. tinctoria* extracts

Plant Extract	IC_{50} mg/ml	TPC mg GAE/100g	TFC mg QE/100g
Leaf	1.08	425± 117.0732	232 ± 0.0111
Stem	2.88	126± 2.134146	32 ± 0.0194
Bark	3.24	179± 50.30488	12 ± 0.00545
Fruit	2.18	182± 28.04878	67 ± 0.0129

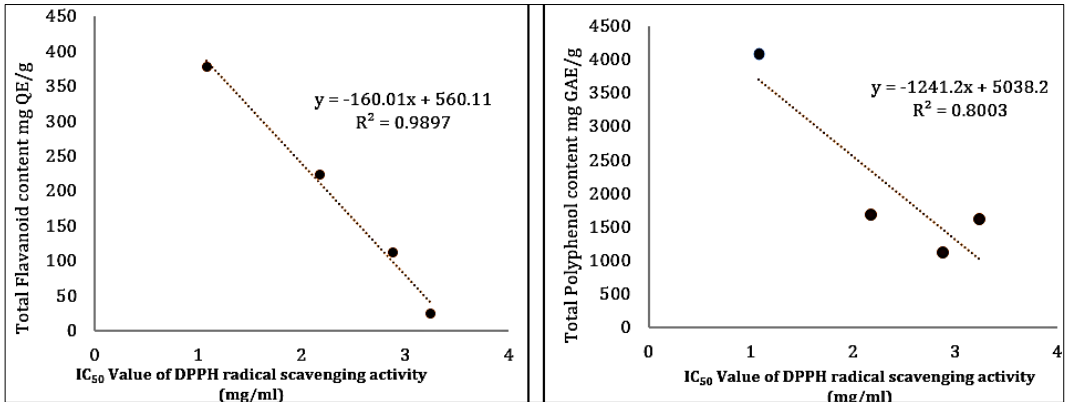

Fig 6: (a) Correlation between DPPH radical scavenging activity (IC_{50}) and total flavonoid (B) Correlation between DPPH radical scavenging activity (IC_{50}) and total Polyphenol

4. Discussion

This study highlights the significant antioxidant potential of various parts of *Wrightia tinctoria* R. Br., as demonstrated by DPPH radical scavenging and Phosphomolybdenum assays. High polyphenol and flavonoid content were observed, particularly in the leaves, which exhibited the strongest antioxidant activity with the lowest IC_{50} value. The strong correlation between total phenolic and flavonoid content with antioxidant activity suggests that these bioactive compounds are crucial in mitigating oxidative stress. The ability of plant extracts to neutralize free radicals, confirmed by both assays, underscores their potential as natural antioxidants, with possible applications in health and pharmaceutical industries.

5. Conclusion

The present study reveals significant antioxidant properties of various plant parts of *Wrightia tinctoria* R. Br. Different plant parts like leaf, stem, bark, fruit contain polyphenols and flavonoids, which correlate with IC_{50} of radical scavenging activity, emphasizing the importance of these compounds in enhancing the plant's bioactivity. DPPH radical scavenging activity and Phosphomolybdenum assay confirmed the ability of different plant extracts to neutralize free radicals and showcase significant antioxidant properties

6. Declaration of Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. Acknowledgement

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