IN-VITRO ANTIOXIDANT ACTIVITY AND ESTIMATION OF FLAVONOID & PHENOLS OF DIFFERENT PARTS OF *DRYOPTERIS JUXTAPOSITA* GROWING IN MUSSOORIE, HIMACHAL PRADESH.

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Abstract

Various diseases are mainly caused by the formation of free radicals, and to counter this, various antioxidants are being used now. The focus of the research is to evaluate the antioxidant activity of the plant extract of Dryopteris juxtaposita, which belongs to the Polypodiaceae family. The total phenolic and flavonoid content are also mentioned in this study. Methods used for antioxidant activity were DPPH assay, FRAP (Ferric Reducing Antioxidant Power) assay, hydroxyl radical scavenging activity, and TAC (Total antioxidant capacity) assay. For the estimation of flavonoids and phenols, aluminium chloride and Folin–Ciocalteu reagents were used, respectively. Two parts of Dryopteris juxtaposita (leaves and stem) were used, among them leaves shown high antioxidant activity then stem. In DPPH assay, leaves were inhibiting 93.36 % of free radicals and in hydroxyl radical scavenging activity it were inhibiting 73.08 % of free radicals at 100 µg/ml concentration. While stem shown 81.17% and 70.59% inhibition of DPPH and hydroxyl radical at same concentration respectively. TAC by phosphomolybdenum reagent and FRAP assay depicted higher antioxidant values in leaves then stem. The total phenolic content was recorded higher in stem than leaves, i.e., 397 mg GAE/g and 159.5 GAE/g respectively whereas, total flavonoids content was 33.66 mg QA/g and 6.06 mg QA/g in leaves and stem respectively. The methanol and water (7:3) extract of Dryopteris juxtaposita was recorded very good natural antioxidant activity having high content of phenolic and flavonoid compounds while comparing with marketed available standard Antioxidant. So, it can be used as an alternative for various diseases caused by free radical.

Key words- Antioxidant; Dryopteris juxtaposita; flavonoids; Phenols

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INTRODUCTION

Antioxidants are compounds that can stop or delay the cell damage that free radicals cause. Free radicals are erratic molecules that are formed by regular body functions as well as external elements including pollution, radiation, and cigarette smoke. Free radicals are neutralizing by antioxidants by giving an electron, which stabilizes them and stops them from harming cells. Foods including fruits, vegetables, nuts, and grains all contain antioxidants. They are essential for preserving general health and chronic illnesses. The most prevalent antioxidants include selenium, beta-carotene, and vitamins C and E.

Dryopteris juxtaposita is an indigenous fern found in India and its neighboring countries[1]. It is a medium-sized, evergreen fern that may grow up to 1.5 meters tall and generally forms clusters pinnate. The leaflets' top surface is glossy and dark green, while their lower side is a lighter shade of green. They are oblong and serrated. On the undersides of the fronds, the fern produces spores that are shielded by kidney-shaped indusia.[2] This fern may thrive in a range of soil types, including sandy loam, clay, and rocky soils, although it prefers a shaded, damp setting. Its lovely foliage and simplicity of care make it a common ornamental plant. Traditional Chinese medicine uses the plant *Dryopteris juxtaposita* to address a variety of ailments, including redness, swelling, fever, and cough.[3] To treat wounds, bruises, and skin irritations, its leaves and roots are frequently applied as a bandage.

The antioxidant activity of the dichloromethane extract of *Dryopteris juxtaposita* was assessed in one research using various assays. The extract demonstrated notable antioxidant activity in scavenging free radicals and preventing the process of lipid peroxidation, which weakens cell membranes. In rats with liver damage brought on by carbon tetrachloride, ethanolic extract of *Dryopteris juxtaposita* was discovered to have potential antioxidant and anti-inflammatory benefits. The extract may have protective benefits against liver damage-based treatments because it was able to lower levels of inflammatory cytokines and oxidative stress indicators.[4]

MATERIAL AND METHODS

Plant collection

Various parts of plant *Dryopteris juxtaposita* were collected form higher altitude region of Mussoorie, Himachal Pradesh[5-7]. After the collection of leaves and stems they were sent for the identification at Botanical survey of India (BSI) (Identification code: 1248). The plant was shade-dried for a week before being powdered into a fine powder and stored at room temperature.

Extract Preparation

Leaves and stem of about 40 gm each in a different Soxhlet apparatus were extracted out with the help of methanolic solvent (METHANOL: WATER) in a ratio of 7:3. The Soxhlet apparatus was run for approximately 6-7 cycles or until it shows colorless solution pass by syphon tube. Then the extract collected from round bottom flask and excess solvent were evaporated utilising a rotary evaporator and stored at room temperature[8-9].

Chemicals used

DPPH (α-diphenyl-β-picrylhydrazyl), ascorbic acid, Sodium Dihydrogen Phosphate, Disodium hydrogen phosphate, Monopotassium phosphate, Dipotassium hydrogen phosphate, Potassium Ferro Cyanide, Trichloroacetic Acid, Ferric Chloride, Tocopherol, Sodium Phosphate, Aluminium Molybdate, Sulfuric Acid, Quercetin, Aluminium Chloride, Sodium Acetate, EDTA (Ethylenediamine tetra acetic acid), Hydrochloric Acid, Hydrogen Peroxide, 2-deoxy-D-ribose, TBA (Tertiary Butyl Alcohol).

Assay by DPPH method

The percentage inhibition of leaves and stem of *Dryopteris juxtaposita* were performed by DPPH assay determined by Kedare and Singh, 2011[10]. DPPH free radical with a violet color that scavenges to a yellow color. This characteristic demonstrates free radical scavenging ability in the DPPH assay. One hydrogen atom will be released during the scavenging reaction between the antioxidant and DPPH, which will then bind to the DPPH-H. DPPH is changed into DPPH-H by antioxidants, which reduces OD levels. The level of discoloration shows how well the antioxidant scavenges free radicals. When antioxidants are present, the DPPH, which was initially violet, turns from yellow to a pale golden color. 0.1 mmol DPPH was dissolved in 100 ml of methanol to make a solution containing 3.94 mg of the compound. Three different plant extracts, ranging in concentration from 20 to 100 µg/ml, were then added to the solution. Then it was vortexed and left for 30 mins. At 517 nm, absorbance was recorded. The same amounts of ascorbic acid were used for the standard. The same procedure was used to perform the blank/control reading, and 517 nm was used to measured absorbance. Percentage inhibition is $(X_0 - X_1)/X_0 \times 100$ where X_0 is Absorbance of the control and X_1 is Absorbance of the sample. After plotting the % inhibition against

the concentration, the IC 50 was determined from the graph.

Assay of Ferric Reducing Antioxidant Power (FRAP)

The method was taken from Rani et al, 2022[11] used to evaluate the capacity of antioxidant to reduce Fe^{3+} ions. To 1 ml of each standard/sample methanol and water (7:3) extract of leaves and stem, 2.5 ml of 0.2 M phosphate buffer with a pH of 6.6 was added. The concentration of the standard which is tocopherol extracts were 20 to 100 µg/ml. The mixture thus prepared was added to 2.5 ml of 1% potassium ferrocyanide which was heated for twenty minutes at 50 °C. After that, centrifugation at 3000 RPM for 5–10 minutes with 2.5ml of 10% trichloroacetic acid (TAC) was performed. Supernatant of about 2.5ml was withdrew and separated out in test tubes, 2.5 ml distilled water was added. After shaking the test tube, 0.5 ml of 10% $FeCl_3$ was added and vortex which led to the formation of blue colored compound. Using a spectrophotometer, the reaction mixture's absorbance was detected at 700 nm. For negative control/blank just take 1ml of methanol and water (7:3 without plant extract) with the same procedure having at same incubation period. Percentage inhibition is calculated by using formula $(X_0 – X_1)/X_0 \times 100$ where X_0 is Absorbance of the control and X_1 is Absorbance of the sample. After plotting the percentage inhibition against the concentration, the IC 50 was determined from the graph.

Hydroxyl Radical Scavenging Activity

The extractives' capacity to scavenge hydroxyl radicals was assessed using the procedure by Moharram et al., 2014[12]. A substance's capacity to scavenge and neutralize highly reactive hydroxy radicals which is a significant cause of oxidative stress and cellular damage is measured by its hydroxy radical scavenging activity. It is thought that substances with high hydroxy radical scavenging activity have therapeutic potential for treating a variety of oxidative stress-related diseases. In this assay, a blend of mixture included 0.8 ml of 50mM phosphate buffer at pH 7.4 added to standard antioxidant (tocopherol)/sample of methanol and water (7:3) plant extracts of leaves and stem of various concentration at 20 to 100 µg/ml. 0.2 ml of 1.00 mM Ethylenediamine tetra acetic acid, 0.2 ml of 1.04 mM $FeCl_3$, and 0.2 ml of 28 mM of 2- deoxy-D-ribose were added to the reaction mixture and adding 0.2 ml of 2 mM ascorbic acid and 0.2 ml of 10 mM H_2O_2 to the reaction. Following that, the test tubes were kept at 37°C for 60 minutes. 1.5 ml of cold Tertiary Butyl Alcohol (TBA) at a concentration of 10 g/L was added after the incubation time was completed. 1.5 ml of 25% HCl was also added. For 15 minutes of heating at 100°C after that the mixture was cooled with water. Utilizing a spectrophotometer, at 532nm, the absorbance of the solution was evaluated. The same process without any plant extract was carried out on the control/blank group. Percentage inhibition is $X_0 – (X_1 – X_2)/X_0 \times 100$, where, X_0 is absorbance of control (without sample) and X_1 is absorbance of sample with 2-deoxy-D-ribose and X_2 is absorbance of sample without 2-deoxy-D-ribose. After plotting the percentage inhibition against the concentration, the IC 50 value was calculated from the graph.

TAC (total antioxidant capacity) by phosphomolybdenum reagent

This method was taken from Adebiyi et al, 2017[13] which was performed by mixing 28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M H_2SO_4 to form the phosphomolybdenum reagent. 1 ml of the phosphomolybdenum solution was added to 0.1 ml of the standard antioxidant (tocopherol)/methanol and water (7:3) extracts of leaves and stem of various concentrations (20 to 100 µg/ml), and the mixture was vortexed. After 1.5 hours of heating the reaction mixture to 90° C, it was cooled for 15-20 minutes to room temperature. At 695 nm, the absorbance was measured, and the result was taken. For the control, 0.1 ml of methanol in 1 ml of phosphomolybdenum reagent was introduced to the test tube. After 1.5 hours at 90° C, absorbance at 695 nm was recorded. Percentage inhibition is $(X_0 – X_1)/X_0 \times 100$, where X_0 is Absorbance of the control and X_1 is Absorbance of the sample. After plotting percentage inhibition against the concentration, the IC50 was determined from the graph.

Determination of Total Flavonoids Content

Estimation of flavonoids by Aluminum Chloride described by the method of Saeed et al, 2012[14]. In this assay, Quercetin was used with different concentration at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml and the volume were makeup with methanol up to 1ml. After introducing chemicals, the reaction mixture was vortexed and diluted by 4 ml by adding methanol. After a quick spin on the vortex, 1ml of 10% aluminum chloride was added which changed the color of the reaction mixture to gold yellow. 1M sodium acetate of 1 ml was added and kept it away from sunlight for about 45 mins. In the end, absorbance was measured at 450 nm and the reading was noted. For the sample preparation, 1ml plant extract was taken in a TT

(without Quercetin) & same procedure was carried out and absorbance was checked at 450nm. The sample's reading was compared to quercetin, and the total amount of flavonoids was calculated as Quercetin equivalents (QAE) which is taken as the milligram content of Quercetin as one gram content of the extract in of dried form.

Total Phenolic Content Analysis

The method was used to determine the total phenolic content of the extracts described by Lu et al, 2011[15]. Gallic acid was used to obtain the calibration curve with conc. of 10, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ml. The procedure started by taking 0.5 ml methanolic gallic acid/methanol and water (7:3) extracts of leaves and stem separately and combined with a reagent of quantity 1 ml (Folin-Ciocalteu), which was taken in ratio of 1:10 volume to volume diluted with water (1:10 v/v). After few mins, 1 ml of Na_2CO_3 having concentration of 75g/ml was mixed in the reaction mixture. After 90 minutes of room temperature incubation, the reaction mixture's absorption at 765 nm was measured. The standard curve was created by graphing absorbance versus concentration. Gallic Acid Equivalent was used to compute the total phenolic concentration using the calibration curve (GAE) (mg of Quercetin per gram of dried extract).

RESULT

In-Vitro enzymic Antioxidant activity

DPPH

In Fig. 1, the methanol and water (7:3) extract of leaves and stem of *Dryopteris juxtaposita* was recorded % inhibition of free radicle DPPH, where the leaves shown 48.40, 75.35, 80.49, 89.09 and 93.36% inhibition of DPPH at 20 to 100 µg/ml respectively. As in stem, at 20, 40, 60, 80 and 100 µg/ml conc. shown 44.71, 67.83, 70.00, 77.24 and 81.17 % inhibition of DPPH respectively. The leaves and stem of *Dryopteris juxtaposita* was compared with standard antioxidant ascorbic acid at 20 to 100 µg/ml which shown 48.40, 51.76, 58.97, 66.19 and 71.63 % inhibition of DPPH free radicle respectively. The IC 50 values of leaves, stem and ascorbic acid was 5.01, 14.73 and 29.15 µg/ml respectively. [mentioned in Fig. 2]

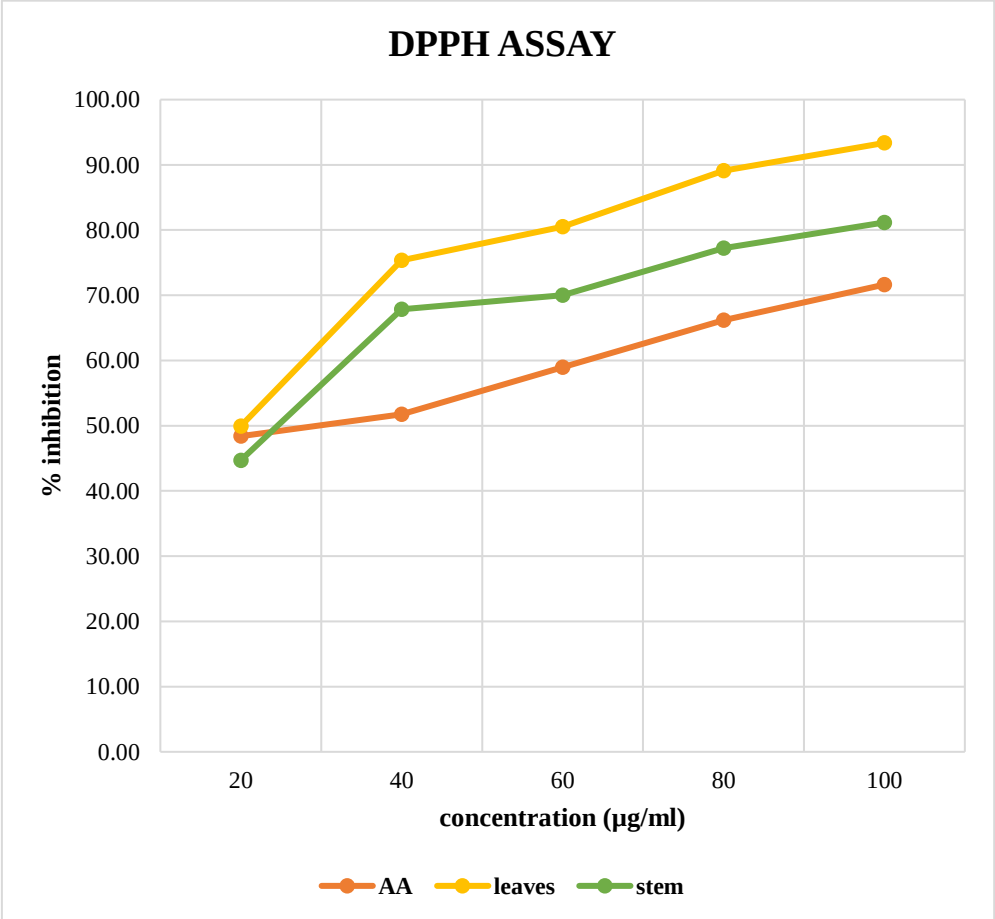


Fig.1 DPPH assay

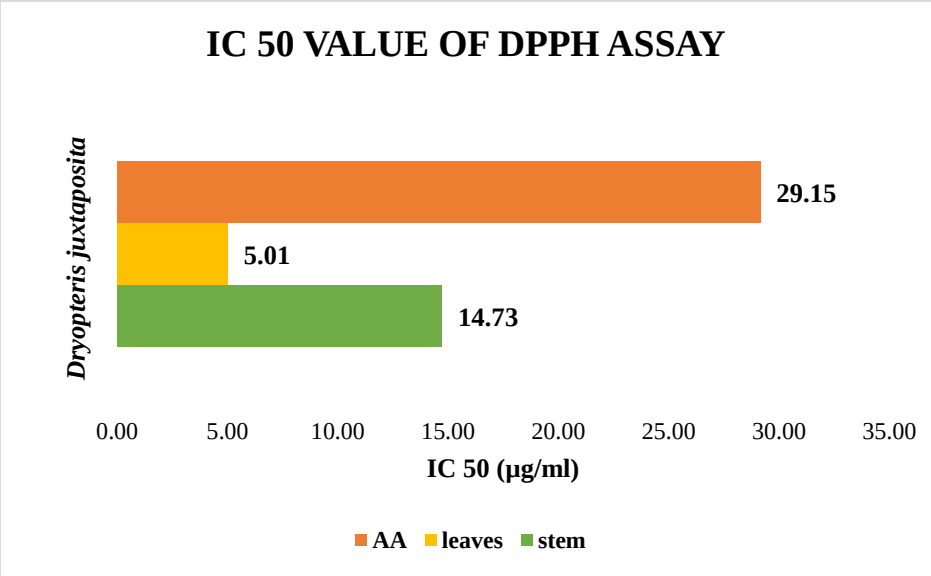


Fig. 2 IC 50 value of *Dryopteris juxtaposita* (Leaves and Stem) and Standard Ascorbic Acid for DPPH assay

Determination of FRAP assay

The antioxidant activity of plant extract of leaves and stem was compared with the standard antioxidant tocopherol. The % inhibition of plant and stem was noted and compared with tocopherol. In Fig. 2, At 20 to 100 µg/ml conc. of leaves extract was recorded 49.33, 70.70, 78.57, 85.69 and 89.62 % of inhibition respectively, while stem extract was 48.16, 61.94, 67.82, 71.02 and 75.23 % of inhibition respectively which is depicted fig. 3. These extractions were compared to the marketed-standard antioxidant tocopherol at concentrations of 20 to 100 µg/ml. The % inhibition were 47.00, 59.74, 62.17, 67.57, and 71.00, respectively. The IC 50 values of leaves, stem and tocopherol was 8.13, 13.08 and 18.817 µg/ml respectively. [mentioned in fig. 4]

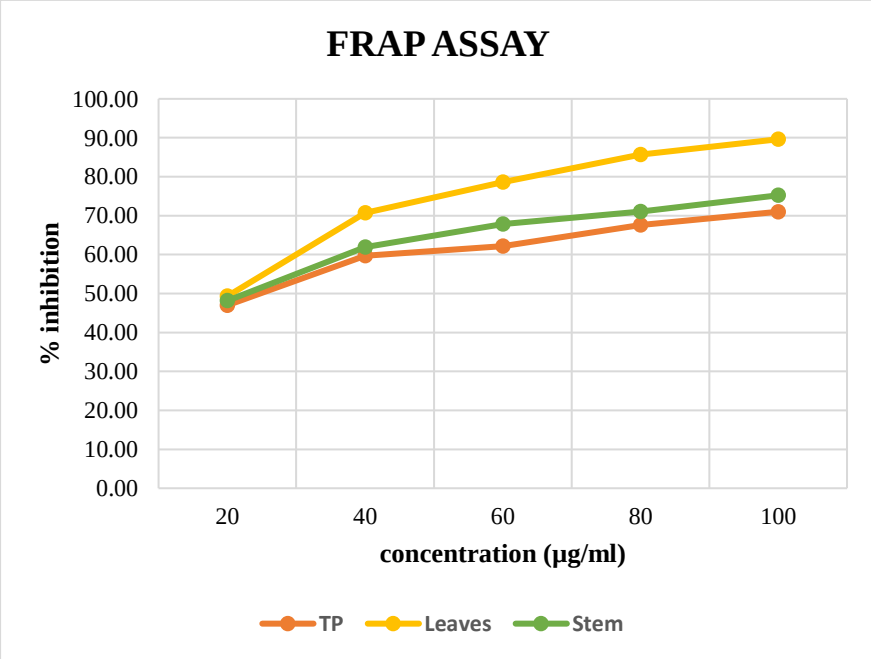


Fig. 3 Determination of FRAP assay

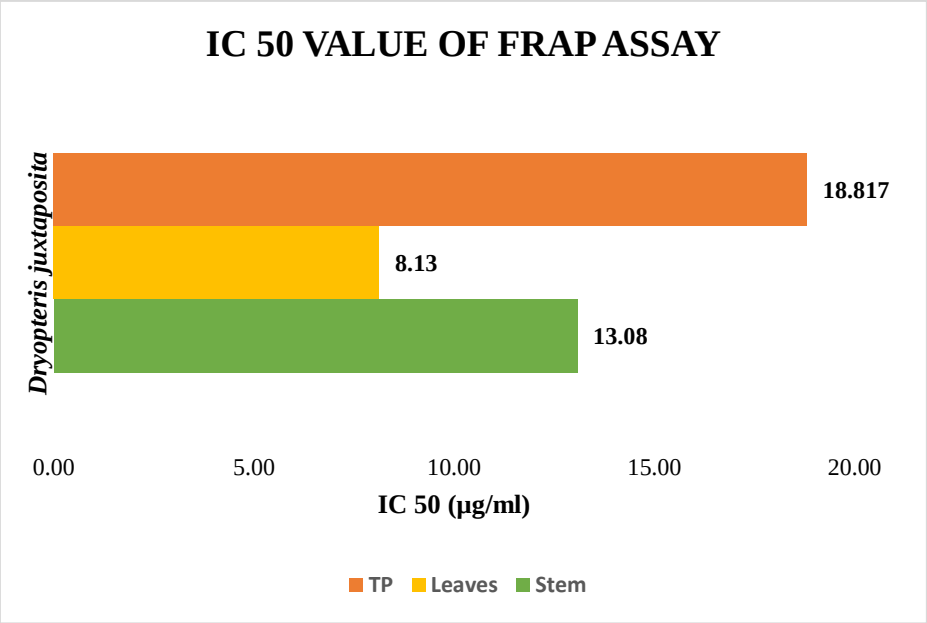


Fig. 4 IC 50 value of *Dryopteris juxtaposita* (Leaves and Stem) and Standard tocopherol for FRAP assay

Determination of TAC

Whereas in TAC assay, which is total antioxidant capacity by phosphomolybdenum reagent, the extract of leaves and stem was compared with two standard antioxidant ascorbic acid and tocopherol. The data is depicted in fig. 5. At 20 to 100 µg/ml concentration of leaves extract, stem extract, ascorbic acid and tocopherol where leaves extract shown 49.41, 78.66, 85.88, 91.42 and 98.00 % of inhibition, stem extract shown 48.41, 60.87, 64.97, 71.25 and 75.00 % of inhibition, ascorbic acid shown 49.78, 51.60, 56.22, 59.40 and 65.00 % of inhibition and tocopherol shown 50.33, 67.90, 71.28, 75.86 and 78.00 % of inhibition respectively. The IC 50 values of leaves, stem, ascorbic acid and tocopherol was 4.21, 15.66, 26.51 and 1.01 µg/ml respectively. [mentioned in fig. 6]

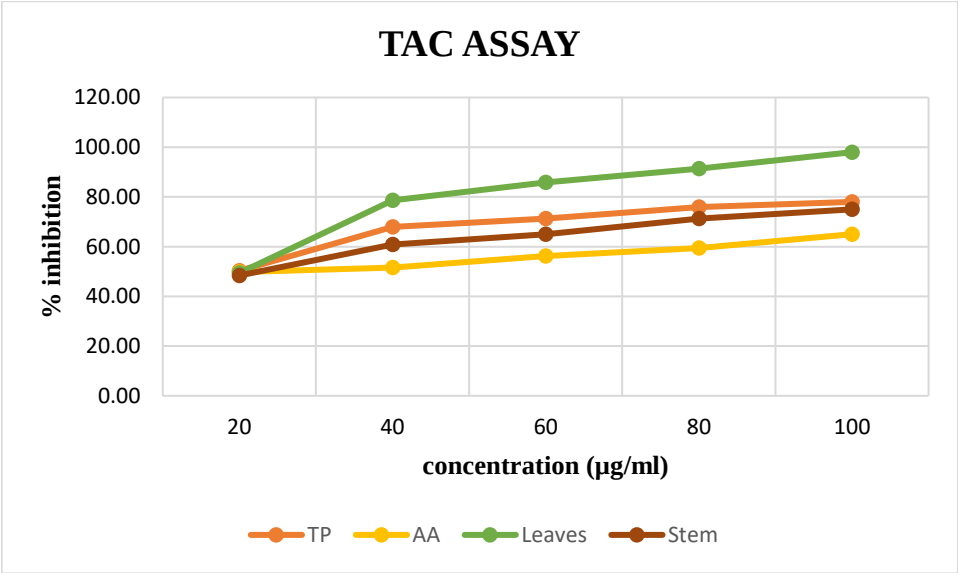


Fig. 5 Determination of TAC assay

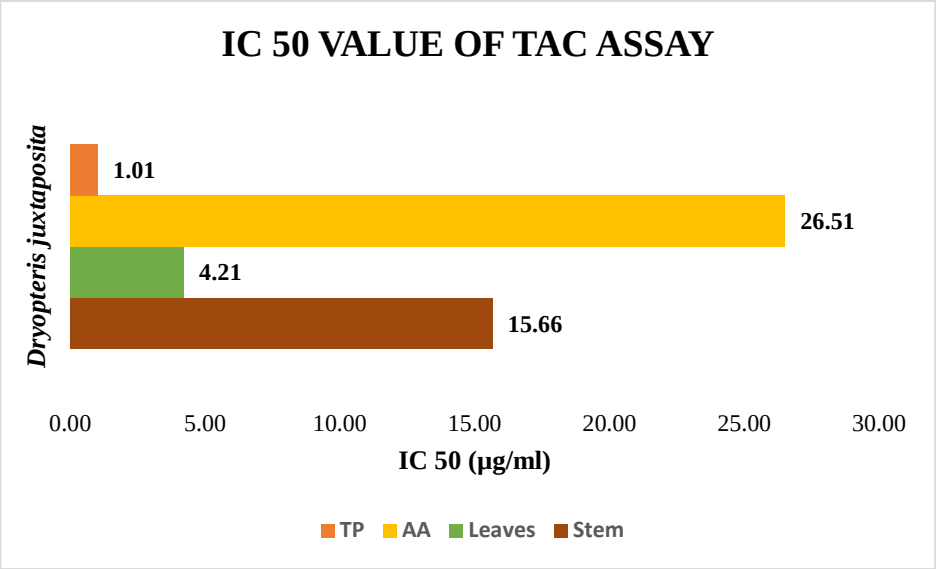


Fig. 6 IC 50 value of *Dryopteris juxtaposita* (Leaves and Stem), Standard antioxidant (Ascorbic acid and tocopherol) for TAC assay

Hydroxyl radical scavenging activity

Plant and leaves extract of *Dryopteris juxtaposita's* capacity to scavenge and neutralize highly reactive hydroxy radicals was measured in terms of % inhibition. Leaves extract shown 52.93, 59.06, 63.26, 68.43 and 73.08 % whereas stem shown 48.13, 58.61, 63.66, 67.75 and 70.59 % inhibition of hydroxy radicals at 20 to 100 µg/ml conc. respectively. These extracts were compared with tocopherol which was a standard antioxidant shown 51.65, 60.82, 68.30, 74.39 and 76.76 % inhibition of free radical at 20 to 100 µg/ml conc. respectively. Shown in Fig. 7. The IC 50 values of leaves, stem and tocopherol were 6.22, 16.54 and 8.64 µg/ml respectively [mentioned in fig. 8].

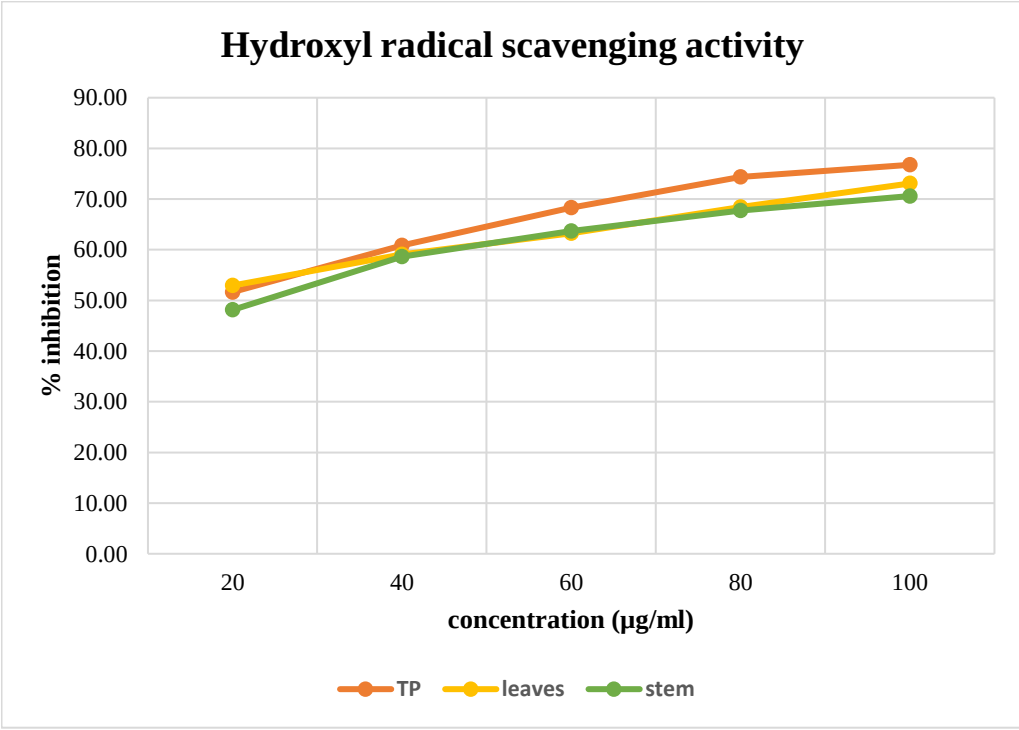


Fig.7. Hydroxyl radical scavenging activity

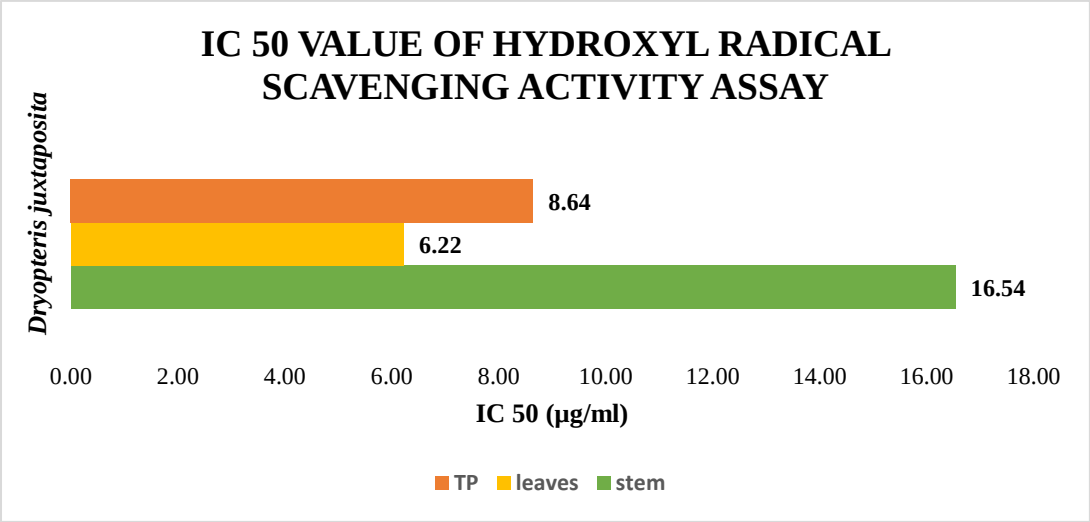
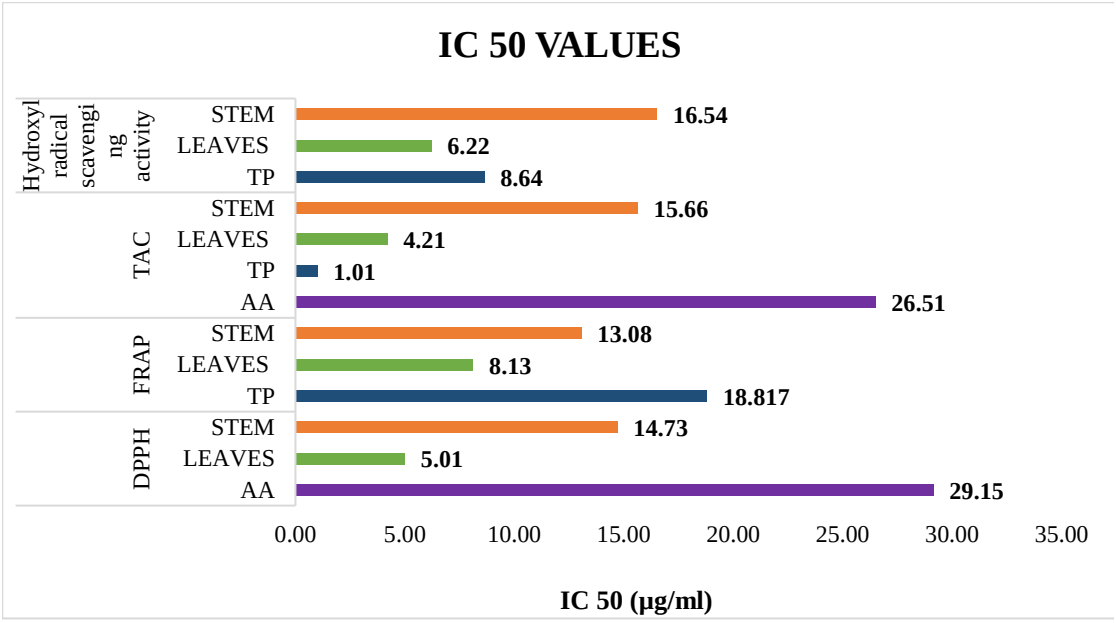


Fig. 8 IC 50 value of *Dryopteris juxtaposita* (Leaves and Stem) and Standard tocopherol for Hydroxyl radical scavenging activity assay

Comparison of IC 50 value between DPPH, FRAP, TAC and Hydroxyl radical scavenging activity

Leaves of the *Dryopteris juxtaposita* were showing lesser IC 50 value then stem which indicated better potency in leaves. All these data were compared two Standard Antioxidant, i.e., Ascorbic acid and Tocopherol. Shown in fig.



Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC).

The total phenolic content present in methanol and water (7:3) extract (leaves and stem) was calculated by Gallic Acid Equivalent/g (mg of Gallic Acid/grams of dried extract). The standard curve equation which was y = 0.0004x + 0.2702, recorded 159.5 mg GAE/g of dried leaves extract while it was 397 mg GAE/g of dried stem extract.

Whereas, total flavonoid content was calculated by Quercetin Equivalent (mg of Quercetin/grams of dried extract). Standard curve equation was y = 0.0071x + 0.306**.** 100% methanol and water (7:3) extract of Leaves and stem was recorded 6.06 and 33.66 mg QAE/g respectively which is depicted in Fig. 10

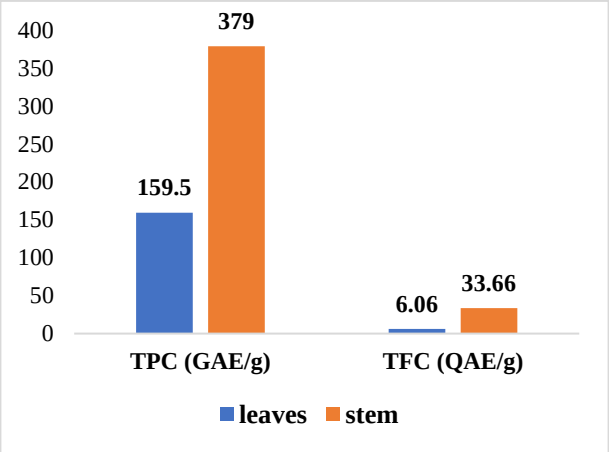


Fig. 10 TPC and TFC of *Dryopteris juxtaposita*

CONCLUSION

In order to keep our bodies healthy and functioning properly, antioxidants are essential. They are substances that defend against oxidative stress, which can harm DNA, proteins, and cell walls. Free radicals are neutralized by antioxidants, which stops them from causing harm. They finish by introducing an electron into the free radical, which stabilizes it and stops it from causing further damage. A few meals that contain antioxidants include fruits, vegetables, nuts, and whole grains. Among the most well-known antioxidants are selenium, vitamin C, vitamin E, beta-carotene, and vitamin E.

In this study, it is concluded that leaves are having better antioxidant activity then Stem compared to marketed standard antioxidant. Various standard antioxidants were used in this study like Ascorbic Acid and Tocopherol. In DPPH assay leaves at 100 µg/ml conc. showing 93.36 % inhibition of free radicals while stem was showing 81.17% and the standard Ascorbic Acid was below 71.63%. In Hydroxyl radical scavenging activity, leaves were showing approximately equal to standard's % inhibition which was tocopherol but stem was showing slightly less % inhibition of about 70.59 %. FRAP and TAC assays were depicted % inhibition of leaves compared to standard antioxidant Tocopherol and Ascorbic Acid. The total phenolic and flavonoid content was measured in terms of GAE/g and QAE/g respectively. In leaves, TPC was 159.5 GAE/g and in stem 397 GAE/g while TFC was 6.06 QAE/g and 33.66 QAE/g in leaves and stem respectively. Stem was showing greater TPC and TFC the leaves. In DPPH assay IC 50 values of leaves and stem were lesser than standard Ascorbic Acid. In Hydroxyl radical scavenging activity leaves were showing lesser IC 50 value than standard antioxidant tocopherol, while stem were showing higher IC 50 value compared tocopherol.

According to the information, *Dryopteris juxtaposita* is a plant that contains a high concentration of natural antioxidants and can be employed in a variety of alternative treatments for diseases caused by free radicals.

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