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Estimation of total Phenolic, Flavonoid Content and Antioxidant Activity of Endophytic Fungi associated with *Centella asiatica* (L.) Urb.

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ABSTRACT

The current investigation aimed to elucidate the free radical scavenging activity of *Centella asiatica* (L.) Urb. and its endophytic fungi. It is a perennial herb, widely used in ayurvedic practices, and has increased over the years basically due to its beneficial functional properties. Its potential antioxidant effect is due to its bioactive constituents. The present study deals with total phenolic content, total flavonoid content, and free radical scavenging activity. The free radical scavenging activity of the plant and its five isolated endophytic fungal extracts were estimated using DPPH and FRAP assays. The total phenolic and total flavonoid content was found to be higher in the hydroalcoholic extract than the aqueous extract of *Centella asiatica* (L.) Urb. while in endophytic fungal extract, *Penicillium sp.* (F2) and *Aspergillus sp.* (F3) showed the highest amount of total phenolic and total flavonoid content respectively. The results of the antioxidant assay indicated that the hydroalcoholic extract of *Centella asiatica* (L.) Urb. exhibited greater free radical scavenging activity than the aqueous extract, while *Aspergillus sp.* (F3) exhibited the highest scavenging activity among the ethyl acetate endophytic fungal extract. During the present work, the highest FRAP value of hydroalcoholic extract was observed while among endophytic fungal extracts, *Aspergillus sp.*(F3) has the highest FRAP value. Thus, in the present study, it can be concluded that the major contributor for antioxidant activity in each assay was the total flavonoid content present in extracts. So, the metabolites from fungi and their host plants are an excellent source of natural antioxidants and efficient against diseases that pose a threat to human health. Hence, these can be used for novel drug formulations.

KEYWORDS- Antioxidant activities, DPPH assay, FRAP assay, Phenolics, Flavonoids

1.INTRODUCTION-

Centella asiatica (L.) Urb. commonly known as Indian Pennywort, belongs to the family Apiaceae. It is a creeping perennial herb that frequently grows in Indian environments and has been traditionally used to treat a variety of ailments and has several distinct biochemical components that are crucial to its use in medicine and nutraceuticals. It has been used to treat skin conditions and has anti-inflammatory, antioxidant, wound-healing, and memory-boosting properties [1]. Also, it has been shown to have a protective effect against oxidative damage caused by neurotoxicity induced by lead acetate [2].

Medicinal plants are a reservoir of endophytes with novel metabolites of therapeutic significance. Research on natural commodities can greatly benefit from the endophytes of medicinal plants [3]. Endophytes comprise a significant amount of the diversity of microorganisms [4]. It is more likely that

endophytic fungi isolated from medicinal plants will exhibit pharmacological potential [5]. Endophytes produce an extensive range of bioactive, structurally distinct natural compounds. These abundant natural products are a huge reservoir with an abundance of unexplored potential for industrial, agricultural, and medical applications [6]. Endophytic fungi have attracted a lot of attention because they are potential sources of new, physiologically active compounds [7,8]. It is feasible to use the secondary metabolites produced by endophytes associated with medicinal plants for medical illnesses [9]. The present study focuses on the total phenolic content, total flavonoid content as well as antioxidant properties of *Centella asiatica* (L.) Urb. plant extract and its five isolated endophytic fungal extracts.

2. MATERIALS AND METHODS

2.1 Sampling-

Centella asiatica plants with no visible symptoms of the disease were carefully selected and collected from the local area of Ranchi. The plant was identified and authenticated by Botanical Survey of India, Kolkata with specimen no. RU/SJM-01.

2.2 Extract Preparation

2.2.1 Plant extract preparation-

Plant samples were collected and rinsed with double distilled water to remove dust and dirt particles then they were washed with 10% saline solution and then 2-3 times with distilled water, then shade dried and ground to powder form and sieved for uniformity. During this experimentation, cold maceration was carried out to get the plant extracts. The hydro-alcoholic extracts of *C. asiatica* were extracted using 70% aqueous and 30% ethanol upon gentle shaking at 120 rpm and 37°C for 72 hours. It was then filtered using Whatman No. 1 filter paper and the solvent was evaporated using rotary evaporator to get the dried extracts. Then it was re-dissolved in two solvent that are aqueous and hydro-alcohol [10].

2.2.2 Fungal extract preparation-

For the mass cultivation of fungus, agar blocks of actively growing pure cultures (3mm in diameter) were placed on 100 ml Potato Dextrose Broth in 250 ml Erlenmeyer flask was incubated at 25±2°C for 3 weeks with periodical shaking at 150 rpm in incubator shaker. Then the culture was filtered through three layers of muslin cloth to remove fungal mycelia. The culture filtrate was then filtered thrice with equal volumes of solvent ethyl acetate. The organic phase was collected and the solvent removed by evaporation under reduced pressure at 45°C using rotatory vacuum evaporator. The dry solid residue was re-dissolved in ethyl acetate and evaluated for its antioxidant activities [11].

2.3 Estimation of phenolic content

The total phenolic content of plant extract and its endophytic fungal extract was estimated using Folin Ciocalteu (FC) reagent, with Gallic acid as the standard. 0.5ml of extract was mixed with 1ml 2N of FC reagent and 4ml of double distilled water in a test tube kept for 5minutes to which 1.5ml of 20% sodium bicarbonate solution was added and mixed thoroughly. The reaction mixture was allowed to stand for 2hours in dark conditions at room temperature. After incubation, absorbance was measured at 765nm with gallic acid taken as standard. The total phenolic content was estimated using a standard calibration curve of gallic acid with different concentration and its working solution prepared in 50% aqueous methanol [12,13,14].

The phenol content was expressed in mg Gallic acid equivalent/gram of extract (mg/gm).

$$\text{TPC}\ \frac{(\text{mg GA})}{\text{gm extract}} = \text{C x V/M}$$

2.4 Estimation of flavonoid content

The total flavonoid contents of plant extract and its endophytic fungal extract was estimated by Aluminium chloride colorimetric method. Different concentrations (20,40,60,80,100&200 µg/ml) of quercetin taken as standard was used for calibration standard curve for estimation of total flavonoid content. 1ml of extract was mixed with 4ml of absolute methanol and 0.3ml of 5% sodium nitrite. After 5min, 0.3ml of 10% Aluminium chloride was added to this reaction mixture followed by the addition of 2ml 1M NaOH after 5min. and the total volume was made up to 10ml with distilled water. The reaction mixture was mixed thoroughly and incubated for 45 minutes. After incubation,

absorbance was measured against a prepared reagent blank at 415nm [15,16,17].

The flavonoid content was expressed in mg Quercetin equivalent/gram of extract(mg/gm)

$$TFC \left(\frac{mg\ Q}{gm\ extract} \right) = C \times V/M$$

2.5 Antioxidant assay

2.5.1 DPPH assay-

The antioxidant activity of the hydro-alcoholic extracts of *C. asiatica* was investigated by DPPH method according to Shimada et al.,1992 with slight modifications [18]. 50µl of aqueous and hydro-alcoholic plant extract as well as 50µl of five different endophytic fungal extract were taken and volume adjusted to 2ml with absolute ethanol. 1ml of 0.1mM ethanolic solution of DPPH was then added to these tubes. The mixture was shaken vigorously and incubate at room temperature for 30minutes in dark. Finally, the absorbance of the solution was measured at 517 nm using a UV-Vis Spectrophotometer [19,20,21].

The percentage inhibition of DPPH radical by the sample extract was calculated using the following relation:

$$\text{Radical Scavenging Activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

A standard calibration curve of DPPH assay for different (0.025M to 1.0M) concentrations of Ascorbic acid was carried out. The percentage of inhibition was plotted against a range of Ascorbic acid standards. The result was calculated as equivalent DPPH activity (equivalent to Ascorbic acid standard in Molar).

2.5.2 FRAP assay-

Three reagents were prepared: Reagent A- 0.1M Acetate buffer, Reagent B-10mM TPTZ(2,4,6-Tris(2-pyridyl)-1,3,5-triazine) solution in 40mM HCl and Reagent C-20mM Ferric Chloride) solution. 0.054gm Ferric Chloride dissolved in 10ml of distilled water. The FRAP reagent was prepared freshly by mixing the reagents (Acetate buffer: TPTZ solution: Ferric Chloride in proportion of 10:1:1(v/v) respectively) and was warmed to 37°C in oven prior to use. 1ml of double distilled water and 1ml of test sample was taken in a test tube. Then 2ml of pre-incubated FRAP reagent was added and were then incubated at 37°C for 60 minutes. Then absorbance was measured at 593nm using UV Visible Spectrophotometer. A standard 2mM Ferrous sulphate was prepared of different Fe^{2+} concentrations of the samples [21,22].

3. RESULTS

3.1 Total phenolic content

Total phenolic content found to be higher in the hydroalcoholic extract (13.296 mg GA/gm) than aqueous extract (7.547 mg GA/gm) of *Centella asiatica* (L.) Urb. when expressed gallic acid as equivalent i.e. mg gallic acid/gm dry weight. Among the endophytic fungal extracts analysed, *Penicillium sp.* (F2) was found to contain maximum phenol content i.e. 5.413 followed by *Aspergillus sp.*(F3) contains 3.291 mg GA/gm and *Curvularia sp*. (F5) was found to be contain 2.127 mg GA/gm. (Graph 1& 2)

3.2 Total flavonoid content

Total flavonoid content was found to be maximum in hydroalcoholic extract (76.503 mg Q/gm) than aqueous extract (60.633 mg Q/gm) of *Centella asiatica* (L.) Urb. when expressed Quercetin as equivalent i.e. mg Q/gm extract. Among endophytic fungal extracts analysed, *Aspergillus sp.* (F3) was found to contain maximum flavonoid content (51.107 mg Q/gm) followed by *Penicillium sp.* (F2) with 27.300 mg Q/gm and *Curvularia sp.* (F5) was found to be contain 23.487 mg Q/gm. (Graph 3&4)

3.3 DPPH assay-

DPPH free radical method is an antioxidant assay based on electron-transfer that produces a purple solution in ethanol. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 517nm, induced by antioxidants. It is visually noticeable as a change in colour from purple to yellow. From the standard curve it was observed that as the concentration of DPPH increases the capacity of free radical scavenging activity also increases. It can be seen that lowest scavenging activity is in 0.025M DPPH i.e. 21.56% and highest

is in 1.0M DPPH i.e. 72.86%. It was observed that free radical scavenging activity of hydroalcoholic extract of *Centella* show highest scavenging activity of 63.50% than aqueous extract which showed 47.15% whereas among endophytic fungus, *Aspergillus sp.* (F3) showed highest scavenging activity of 37.83% followed by *Penicillium sp.* having 32.32% scavenging activity. (Graph 5 & 6)

3.4 FRAP assay-

FRAP assay is based on the rapid reduction in Ferric-tripyridyl triazine (Fe^{III}-TPTZ) to Ferrous-tripyridyl triazine (Fe^{II}-TPTZ), a blue coloured product by the presence of antioxidants in extracts. The antioxidant activities were expressed as the concentration of antioxidant having a ferric reducing ability equivalent to that of 2mM of $FeSO_4$. A standard curve was created by adding the FRAP reagent to a range of Fe^{3+} solutions of known concentrations which allows the Fe^{3+} concentration of the samples to be calculated thereby determining antioxidant capacity. FRAP value for aqueous and hydroalcoholic extract of *Centella asiatica* (L.) Urb. was determined and it was found to be 0.682 for hydroalcoholic extract and 0.589 for aqueous extract. Among endophytic fungal extract, *Aspergillus sp.*(F3) has highest FRAP value of 0.469 followed by *Penicillium sp.*(F2) has FRAP value of 0.442 and FRAP value for *Curvularia sp.*(F5) was found to be 0.344.(Graph 7 &8)

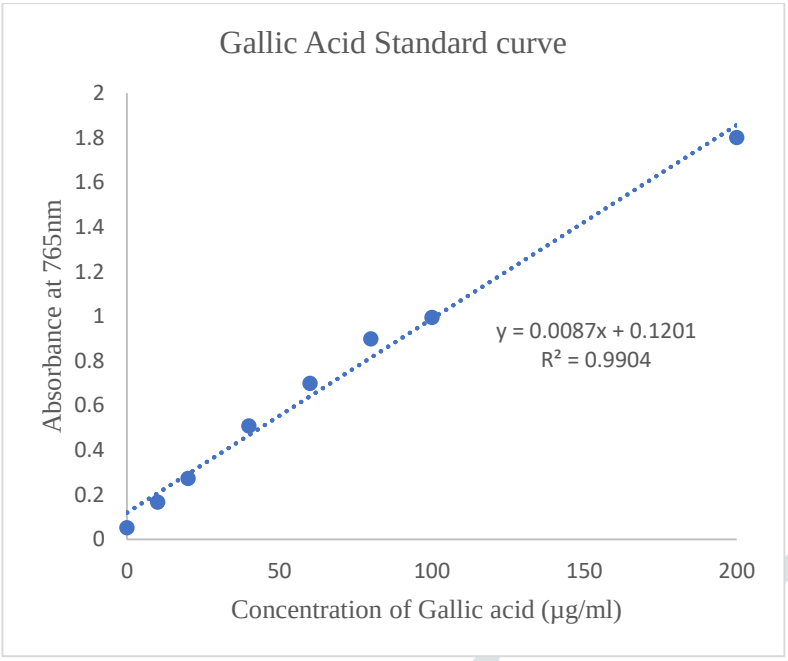
4. DISCUSSION-

The past few decades have seen a significant increase in the medicinal use of plants. A wide range of secondary metabolites having antioxidant potential are synthesized by plants. Antioxidants inhibit the effects of free radicals, which have been associated with the aging process and the causes of many diseases [23,24,25]. Microbes with a wide range of varieties that are still investigated for metabolite production may possess valuable bioactivities, including antioxidant activity [26]. There is a substantial correlation between total phenol content, flavonoids, alkaloids, and antioxidant activity, as phenols possess strong scavenging abilities for free radicals due to their hydroxyl groups [27]. Consequently, it has been reported that the phenolic content of plants may directly contribute to their antioxidant effect [28,29,30]. Phenolic compounds are powerful chain-breaking antioxidants and are reported to be associated with antioxidant activity, which plays a crucial role in stabilizing lipid peroxidation [31].

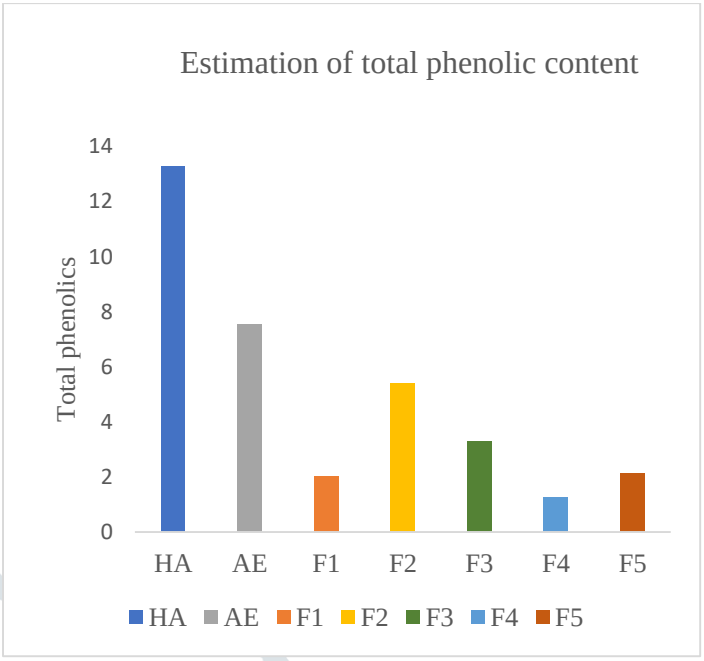
Total phenol content was quantified in the current study using a spectrophotometric method with gallic acid as a standard. Quantitative estimation of total flavonoids was done by an aluminium chloride assay. The highest phenol and flavonoid content were observed in the hydroalcoholic extract of *Centella asiatica* (L.) Urb, having a phenol content of 13.296 mg GA/gm and flavonoid content of 76.503 mg Q/gm. However, the ethyl acetate endophytic fractions of plants exhibited phenol and flavonoid content, with *Penicillium sp.* having a phenol content of 5.413 mg GA/gm and *Aspergillus sp.* having a flavonoid content of 51.107 mg Q/gm, respectively.

In the present study, the results indicate that the hydroalcoholic extract exhibited the highest FRAP value as well as free radical scavenging activity compared to the aqueous extract. The ethyl acetate extracts of five fungal endophytes were subjected to antioxidant activity assays by both FRAP and DPPH free radical scavenging assays. Free radical scavenging activity of hydroalcoholic extract showed the highest scavenging activity than aqueous extract of *Centella asiatica*. Among the endophytic fungal extract, *Aspergillus sp.* (F3) showed highest scavenging activity followed by *Penicillium sp.* (F2). It was noted that the endophytic fungi produced the highest activity by the FRAP assay also showed the highest free radical scavenging activity by the DPPH assay. It was observed that the fungal endophytes that had the highest flavonoid content were also found to possess the highest free radical scavenging activity. It can be concluded that the presence of flavonoids as well as phenolic compounds plays a role in the enhancement of antioxidant activity.

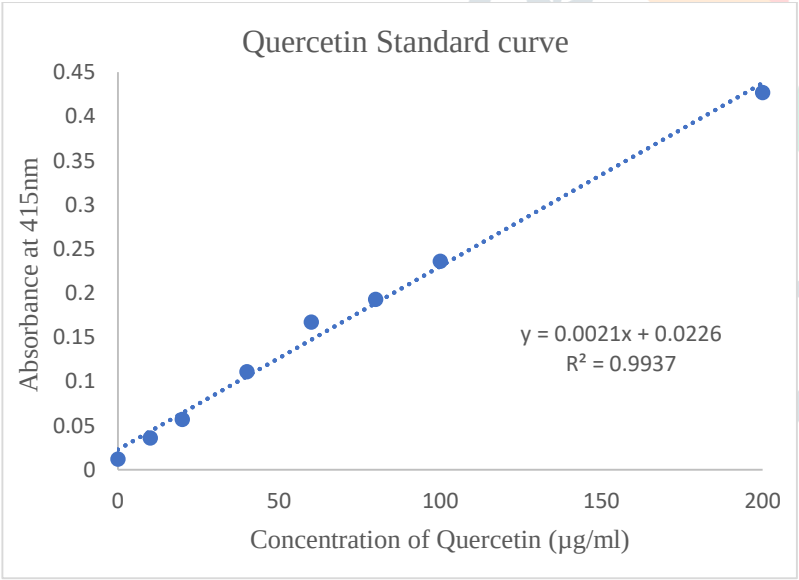
Considering the total phenolics, total flavonoid, FRAP assay, and DPPH radical scavenging activity as indices of the antioxidant activity of the extracts, the present findings reveal the potential of the extract as a source of natural antioxidants [32].



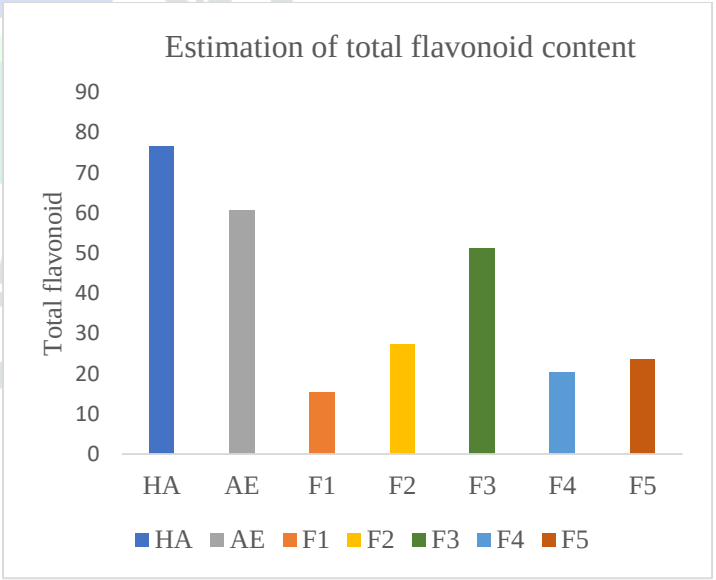
Graph 1- Total phenolic content in standard Gallic acid



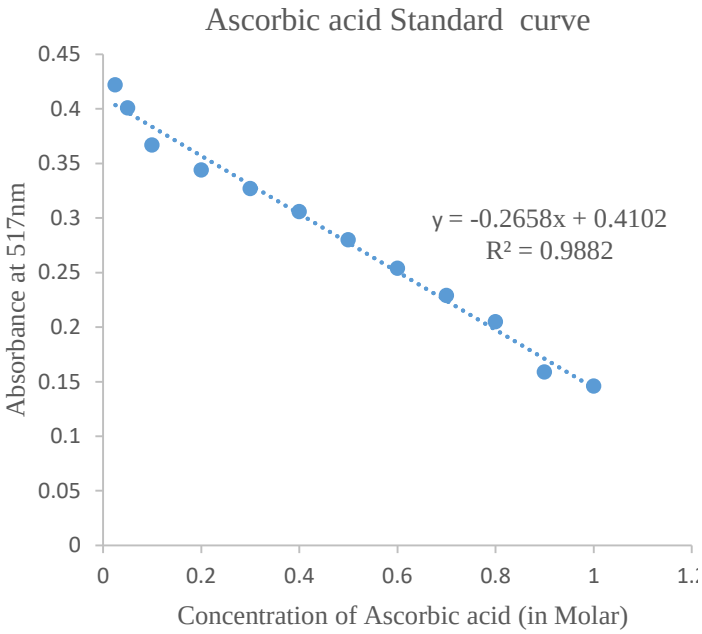
Graph 2- Total phenolic content in plant extracts and its endophytic



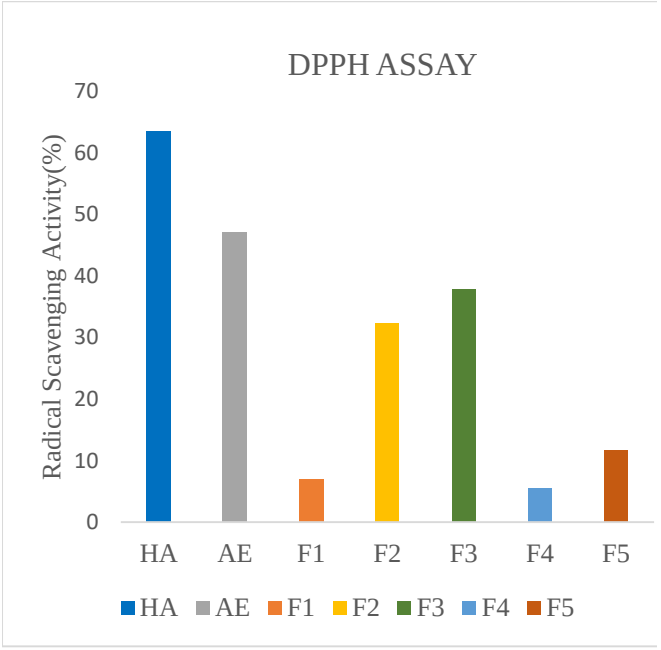
Graph 3- Total flavonoid content in standard quercetin



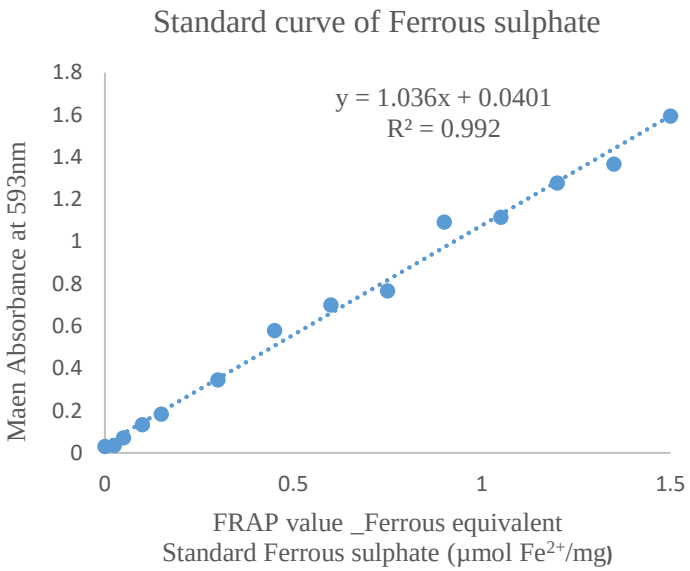
Graph 4- Total flavonoid content in plant extracts and its endophytic fungal extracts



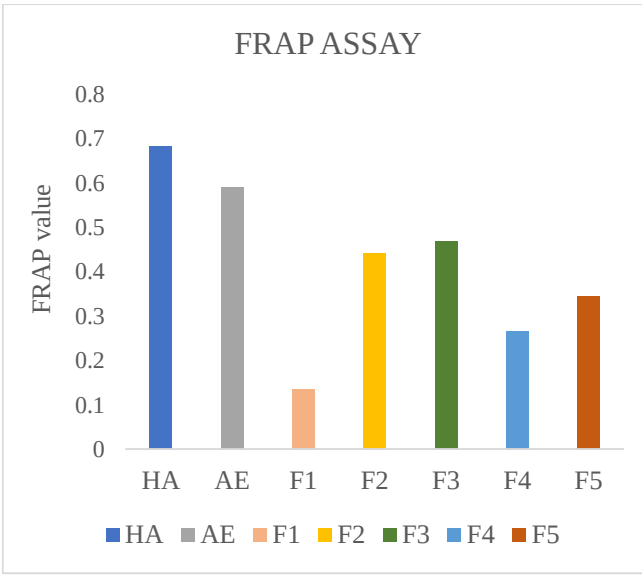
Graph 5- DPPH radical scavenging assay of standard ascorbic acid



Graph 6- DPPH radical scavenging assay of plant extract and its endophytic fungal extract



Graph 7- FRAP value of standard Ferrous sulphate ($FeSO_4.7H_2O$)



Graph 8- FRAP value of plant extracts and its endophytic fungal extracts

5. CONCLUSION

In this present investigation, the total phenolic and total flavonoid contents were estimated and the biological activities were also analyzed by DPPH and FRAP assay. It was observed that the fungal endophytes that had the highest flavonoid content were also found to possess the highest free radical scavenging activity. It can be concluded that the presence of flavonoids as well as phenolic compounds plays a role in the enhancement of antioxidant activity. Antioxidant activity is higher in plant extract than its endophytic fungal extract.

6. CONFLICTS OF INTEREST-

The authors declare no conflict of interest.

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