



ISSN (E): 2320-3862  
ISSN (P): 2394-0530  
NAAS Rating 2017: 3.53  
JMPS 2017; 5(2): 106-111  
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Received: 12-01-2017  
Accepted: 13-02-2017

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## Total phenolics, flavonoids content and antioxidant activities of some *Eulophia* species

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### Abstract

The aim of the study was to determine total phenolics, flavonoid contents and antioxidant activity in some *Eulophia* species (Orchidaceae). Rhizomes of *Eulophia* species viz. *E. nuda*, *E. ochreatea*, *E. herbacea*, *E. ramentacea*, *E. andamanensis* and *E. epidendrea* were extracted with petroleum ether, methanol, acetone and Distilled water. The antioxidant capacity of the extracts was determined using the ferric reducing antioxidant potential assay, and the free radical-scavenging capacity was evaluated using 2,2-diphenyl-1-picrylhydrazyl radical-scavenging. Maximum amounts of total phenolics and antioxidant activity were observed in methanol extracts of all *Eulophia* species studied. Acetone proved to be best solvent for extraction of flavonoids in all *Eulophia* species. Among the species studied, *E. nuda* showed maximum yield of total phenolic contents, flavonoid contents, and antioxidant activity. The study also revealed that there is strong correlation in phenolics and antioxidant activity.

**Keywords:** *Eulophia* species, antioxidant activity, DPPH, phenolics, flavonoids

### 1. Introduction

Diverse plant resources are being exploited for their healing and promoting health effects in human being. The discovery of natural and safe sources of antioxidants of plant origin has increased markedly in recent years, and they have been recognized to be a better alternative than synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, and others. However, considering the wide range of plant resources available in the world, many of them still remain unexplored.

The genus *Eulophia* (Orchidaceae) includes mostly terrestrial species and distributed worldwide. In various states of India, rural and tribal communities such as Bhill, Garasia, Gujjar, Kathodi, Korkus, Koyas, Meena, Oriya, Paliyan, Pardhi, Pawara, Tadvi and Wanjari etc. use ethno-botanical potential of different *Eulophia* species for various ailments especially fertility, aphrodisiac, skin protective, vermifuge, antiscrofulous, anti-bellyache, anti-rheumatic antifatigue, wound healing and antitumour activity [1]. Earlier phytochemical studies showed presence of essential minerals, polyphenols, saponins, alkaloids, phytic acid etc. in *E. ochreatea* and *E. nuda*. Nine different phenanthrenes, a rather uncommon class of aromatic metabolites, have been reported from *E. nuda* till date [2]. An amorphous phenanthrene, named nudol, later identified as 2,7-hydroxy-3,4-dimethoxyphenanthrene has been isolated from *Eulophia nuda*, *E. ohreatea*, *Eria carinata* and *Eria stricta* [3-6]. Phenanthrene derivative 9, 10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol, isolated from rhizomes showed promising cytotoxic activity against human cancer cells [7]. However, there are few reports of antioxidant activity, phenolic and flavonoid compound profiles in *Eulophia* species.

The present study was carried out to evaluate potential of *Eulophia* species with respect to antioxidant activity, total phenolic content, total flavonoid content and correlation between them.

### 2. Material and methods

#### 2.1 Plant material

*Eulophia nuda*, *E. ochreatea*, *E. herbacea* and *E. andamanensis* maintained in Botanical Garden, Dept. of Botany, Shivaji university, Kolhapur (MS), India and *E. ramentacea* collected from Satara, *E. epidendrea* from Kerala, were used for determination of the antioxidant potential of these species. The species were identified by Dr. R. V. Gurav, and herbarium specimen deposited in Dept. of Botany, Shivaji University, Kolhapur (MS), India.

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## 2.2 Extraction of plant material

Rhizomes of *Eulophia nuda*, *E. ochreatea*, *E. herbacea*, *E. epidendrea*, *E. andamanensis*, and *E. ramentacea* were chopped, shed dried and grind to fine powder with mixer. 500 mg of fine powder of each species was extracted separately with 20 ml petroleum ether, acetone, methanol and distilled water at room temperature for 24 hrs. The extract was filtered using Büchner funnel and stored at 4 °C till further use.

## 2.3 Chemicals

All the chemicals and solvents used were of analytical grade. Folin-ciocalteu reagent, sodium carbonate, aluminium trichloride, ferric chloride, potassium acetate, ascorbic acid, Gallic acid, Rutin, 2,4,6- tripyridyl-s-triazine (TPTZ), 2,2-Diphenyl-1- picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co.

## 2.4 DPPH radical scavenging activity

In the DPPH method, the antioxidants react with the stable free radical *i.e.*, 2,2-diphenyl-b-picrylhydrazyl (deep violet color) and convert it to 2,2-diphenyl-b-picrylhydrazine with discoloration. The degree of discoloration indicates the scavenging potentials of the sample antioxidant. The various extracts were measured in terms of hydrogen donating or radical scavenging ability using the DPPH assay [8]. 0.2 ml extracts of each species in different solvents were added in 2.7 ml DPPH (60 µM in methanol) solution. The absorbance was measured at 517 nm on UV –Visible spectrophotometer (Shimadzu UV-1800; Tokyo, Japan) after 30 minutes of reaction in the dark. The percent of DPPH decolorization of the sample was calculated according to the equation:

$$\% \text{ Decolourization} = [1 - (\text{ABS SAMPLE} / \text{ABS CONTROL})] \times 100.$$

## 2.5 FRAP (Ferric Reducing Antioxidant Power) Assay

Like DPPH, FRAP is also a very commonly used antioxidant assay used in the analysis of antioxidant capacities of medicinal plants. It's a very simple, rapid, sensitive and inexpensive approach. The reducing capacity of a compound might serve as a significant indicator of its antioxidant capacity.

The stock solutions contained 300 mM acetate buffer (3.1 g  $\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$  and 16 ml  $\text{C}_2\text{H}_4\text{O}_2$ ), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution and then warmed at 37 °C before using. Extracts (200 µl) were allowed to react with 2700 µl of the FRAP solution for 30 minutes in the dark condition. Absorbance of the coloured product [ferrous tripyridyltriazine complex] was then measured at 593 nm [9]. Results were expressed in mg AAE/g dry mass, compared with those of standard for ascorbic acid.

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine ( $\text{Fe}^{3+}$  – TPTZ) complex and produce a colored complex of ferrous tripyridyltriazine ( $\text{Fe}^{2+}$  –TPTZ).

## 2.6 Determination of total phenolics

Total phenolic contents were determined using Folin-Ciocalteu colorimetric method [10]. Each 0.125 ml extracts mixed with 1.8 ml Folin-Ciocalteu reagent (Sigma) and kept at room temperature for 5min. followed by mixing of 1.2 ml of 15 % (w/v)  $\text{Na}_2\text{CO}_3$ . The mixtures were shaken and allowed to react for 90 minutes in the dark. Absorbance was measured at 765 nm, and gallic acid was used as standard.

The total phenolic content was determined as gallic acid equivalents (GAE)/mg of extract.

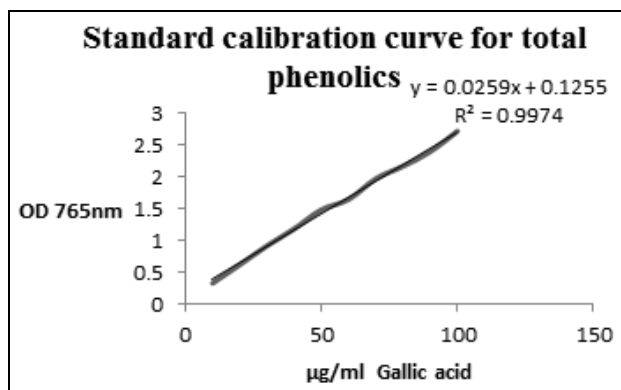


Fig 1: standard calibration curve for total phenolics

## 2.6 Total flavonoid assay

The total flavonoids in each extract were determined by Aluminium Chloride Colorimetric Method [11]. An aliquot (0.5 ml) of extract was added to 1.5 ml methanol; 0.1 ml aluminium chloride, 0.1 ml potassium acetate solution and 2.8 ml distilled water were added and mixed well. Sample blank was prepared in similar way by replacing aluminium chloride with distilled water. The absorbance was measured at 510 nm by using UV-visible spectro-photometer. The results were expressed as rutin equivalents/mg DW.

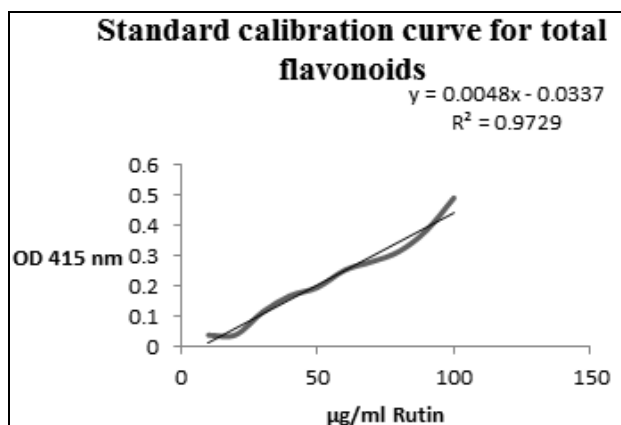


Fig 2: standard calibration curve for total flavonoids

## 2.7 Statistical analysis

Data from this study (samples run in triplicates, n=3) are represented as mean values ± SD. Correlation between different parameters was determined using SPSS ver.21. (IBM SPSS ver 21).

## 3. Results and discussion

### 3.1 DPPH Assay

Reactive oxygen species (ROS) including free radicals (superoxide radical, hydroxyl radical, peroxy radical) and non radical species (hydrogen peroxide, singlet oxygen) are produced by various means such as radiations, chemical reactions and several redox reactions. These ROS are implicated in oxidative stress which is due to imbalance in generation of ROS and antioxidant defense system of the body. These ROS contribute to protein oxidation, DNA damage and lipid peroxidation in living systems and are implicated in various patho-physiological conditions such as cancer, cardiovascular diseases, diabetes, liver cirrhosis,

neurological disorders such as Parkinson's and Alzheimer's disease and others. Cells have several antioxidant defense mechanisms that help in prevention of damaging effect produced by ROS and include antioxidant enzymes viz., superoxide dismutase, catalase, glutathione oxidase and small molecules such as vitamin C and vitamin E. However, in oxidative stress, there is an extra need for antioxidants from exogenous sources. Strong restrictions have been placed on the use of synthetic antioxidants such as BHT, BHA and gallates due to their doubtful safety and potential adverse effects. This led to an increasing interest in natural antioxidants. Plants have been considered as richer sources of antioxidants [12].

DPPH is one of the few, commercially available stable N-centred organic free radical and has an UV-Visible absorption maximum at 515-517nm in methanol. On accepting hydrogen from a corresponding donor, the solution of DPPH loses the characteristic deep purple color and become yellow colored Di-phenyl Picryl hydrazine. DPPH radical scavenging activity is one of the widely used assays to determine antioxidant activity of many compounds including plant extracts [12].

In present study methanol and acetone extracts of all species shows maximum radical scavenging activity (RSA) (Fig 3). Methanolic extract of *E. nuda* showed highest % inhibition (86.28±0.06) while in *E. herbacea* Petroleum ether extract % inhibition was minimum (2.55±0.03).

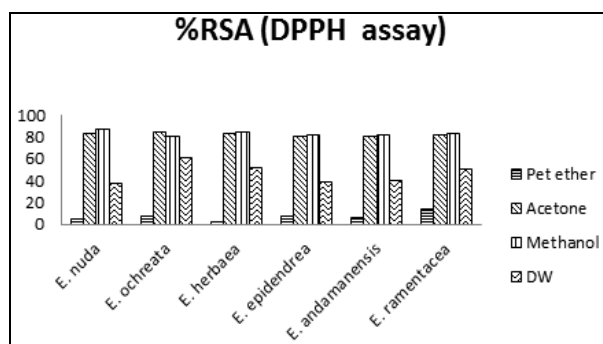


Fig 3: Antioxidant activity in *Eulophia* species (% RSA)

### 3.2 FRAP assay

A number of assays are designed to measure overall antioxidant activity, or reducing potential, as an indication of a host's total capacity to withstand the adverse effect of free radical stress. Reducing power reflects the electron donating capacity of bioactive compounds, and associated with antioxidant activity. The reducing capacity of a compound can be measured by the direct reduction of  $\text{Fe}[(\text{CN})_6]_3$  to  $\text{Fe}[(\text{CN})_6]_2$ . Addition of free  $\text{Fe}^{3+}$  to the reduced product leads to the formation of the intense Perl's Prussian blue complex,  $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ , which has a strong absorbance at 700nm. An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. The ferric ion reducing antioxidant power assay takes the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of its potential antioxidant activity. The

reducing capacity of a compound might serve as a significant indicator of its antioxidant capacity. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine ( $\text{Fe}^{3+}$  – TPTZ) complex and produce a colored complex of ferrous tripyridyltriazine ( $\text{Fe}^{2+}$  – TPTZ).

The reducing ability of various extracts of *Eulophia* species was in range of 1.937±0.34 to 0.210±0.01 mg AAE/g. (Fig. 4). FRAP activity was found to be higher in *E. nuda* (1.937±0.34 mg AAE/g) methanol extract followed by Acetone extract (1.833±0.37). The lowest activity was in *E. herbacea* (0.210±0.01) in petroleum ether extract. Methanol and acetone found to be most appropriate solvents for estimation of FRAP activity.

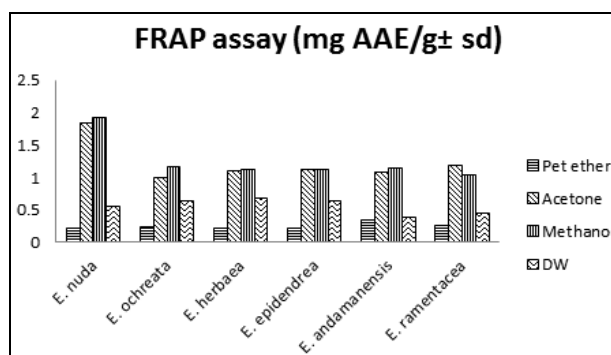


Fig 4: Antioxidant activity in *Eulophia* species (FRAP assay)

It is evident from the present study that the extract possesses reductive potential and could serve as electron donors, terminating the radical chain reactions.

### 3.3 Total phenolic contents of different *Eulophia* species

Phenolics are one group of larger secondary metabolites, which are synthesized by plants and utilized as UV, wounding and infection protectant in plants. Natural phenolic compounds play an important role in cancer prevention and treatment. Phenolic compounds from medicinal herbs and dietary plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others. Various bioactivities of phenolic compounds are responsible for their chemopreventive properties (e.g., antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects) and also contribute to their inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways [13].

The Folin-Ciocalteu (F-C) reagent is sensitive to reducing compounds, polyphenols and thus produces a blue colour complex. The F-C assay relies on the transfer of reducing equivalents (electrons), in the alkaline medium, from phenolic compounds to phosphomolybdic /phosphotungstic acid complexes, manifested in the formation of blue colour complexes [possibly (PMoW11O40) 4-] that are determined on a UV-visible spectrophotometer by monitoring the absorbance at 765 nm.

Table 1: Total phenolic content in *Eulophia* species

Solvent	Total phenolic content (mg GAE/g± sd)					
	<i>E. nuda</i>	<i>E. ochreata</i>	<i>E. herbacea</i>	<i>E. epidendrea</i>	<i>E. ramentacea</i>	<i>E. andamanensis</i>
Pet. Ether	1.29±0.00	0.12±0.00	1.31±0.01	1.22±0.00	0.86±0.00	0.58±0.00
Acetone	7.53±0.05	4.05±0.34	2.51±0.04	6.61±0.08	6.07±0.16	2.31±0.16
Methanol	9.19±0.01	6.15±0.04	5.96±0.01	7.77±0.04	7.42±0.03	6.48±0.02
DW	4.51±0.01	5.03±0.02	4.03±0.13	5.16±0.02	3.30±0.01	1.20±0.01

The amount of total phenolic compounds in different solvent extracts from *Eulophia* species are depicted in table 1. Their gallic acid equivalent (GAE) values were calculated using equation obtained from standard graph of gallic acid (Fig 1 a). The total phenolic content (TPC) was in range of 9.191±0.01 to 0.128±0.00 mg GAE/g. *E. nuda* shows highest phenolic content in methanol (9.191±0.01 mg GAE/g) followed by acetone (7.538±0.05), DW (4.518±0.01) and petroleum ether (1.297±0.00) extracts. The TPC in methanolic extracts of different species varies as *E. nuda*> *E. epidendrea*>*E. ramentacea*>*E. andamanensis*>*E. ochreata*>*E. herbacea*. Lowest TPC was found to be in petroleum ether extract of *E. ochreata* (0.128±0.00 mg GAE/g). Maximum TPC was found in Methanolic extract followed by Acetone> DW> Petroleum ether. The results revealed methanol as best solvent for extraction of phenolic compounds in all species studied, while petroleum ether as least suitable.

### 3.4 Total flavonoid contents of different *Eulophia* species

Flavonoids are universally present as constituents of flowering plants, particularly of food plants. The high intake of foods and beverages rich in polyphenols, especially in

flavonoids, has been associated with decreased risk of neoplasm. Dietary flavonoids inhibit the proliferation of various cancer cells and tumor growth in animal models [14]. Epidemiologic data suggested that flavonoids consumption may protect against cancer induction in several human tissues [15]. Chemoprevention has the potential to be a major component of colon, lung, prostate and bladder cancer control. A number of investigators have reported that flavonoids inhibit the tumour growth by interfering with some phases of the cell cycle [16]. The use of flavonoids for prevention and cure of human diseases is already widespread. These aspects made flavonoids an interesting object for industrial production.

Formation of acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols in addition with aluminium chloride. Aluminium chloride also forms acid labile complexes with the ortho - dihydroxyl groups in the A- or B-ring of flavonoids. For building the calibration curve, rutin is used as a standard material. Various concentrations of standard rutin solution were used to make a standard calibration curve.

**Table 2:** Total flavonoid contents in *Eulophia* species

Solvent	Total Flavonoid content (mg RE/g± sd)					
	<i>E. nuda</i>	<i>E. ochreata</i>	<i>E. herbacea</i>	<i>E. epidendrea</i>	<i>E. ramentacea</i>	<i>E. andamanensis</i>
Pet. Ether	0.32±0.00	0.31±0.00	0.47±0.01	0.37±0.01	0.38±0.01	0.35±0.01
Acetone	6.23±0.12	1.62±0.01	2.46±0.02	3.31±0.02	2.86±0.00	1.68±0.00
Methanol	1.08±0.00	1.17±0.01	1.76±0.01	2.01±0.01	2.26±0.01	1.61±0.01
DW	0.60±0.00	0.69±0.00	0.66±0.00	1.01±0.09	1.36±0.01	0.90±0.01

The amount of total flavonoid content (TFC) was determined in extracts of *Eulophia* species in different solvents (Table 2). From the results, it was revealed that *E. nuda* showed the highest amount of total flavonoid content as Rutin equivalent (RE) (6.23±0.12 mg RE/g) where as *E. ochreata* reported lowest flavonoid content (0.31±0.00 mg RE/g) compared to other *Eulophia* species under study. In all species TFC followed a pattern of solubility as Acetone> Methanol>DW>Petroleum ether. Among all species acetone was found to be best suitable for extraction of flavonoids. The species under investigation showed variation in phenolic and flavonoid content and associated antioxidant activity with respect to polarity of solvents used, suggest that the diverse group of phenolic and flavonoid compounds are present in studied species.

### 3.5 Correlation between TPC, TFC and antioxidant activity in *Eulophia* species

Many investigations revealed that the phenolics and flavonoids content contribute to the antioxidant activities of

plants. Many studies revealed that there is positive correlation between antioxidant activity potential and amount of phenolic compounds of the extracts [17, 18]. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [19]. Plant flavonoids, in general are highly effective free-radical scavengers and antioxidants [20].

Strong positive correlation was observed between antioxidant capacity and TPC in all species studied (table 3) except *E. ochreata*, where linear correlation between TFC and antioxidant capacity as determined by DPPH assay was significant ( $R^2=0.906$ ). In *E. nuda* significant correlation between TPC and DPPH activity was found to be  $R^2=0.961$  and TPC with FRAP assay  $R^2=0.974$  implying that phenolic compounds contributing to antioxidant activity possessed by species. The flavonoid content least participating for its radical scavenging activity as evident from its correlation with FRAP and DPPH ( $R^2=0.467$  and  $R^2=0.487$  respectively.).

**Table 3:** Correlation between TPC, TFC and antioxidant activity in *Eulophia* species

Correlation	<i>E. nuda</i>			<i>E. ochreata</i>		
	p value	Remark	$R^2$	p value	Remark	$R^2$
Phenolics vs Flavonoids	0.804	Non-significant	0.645	0.499	Non-significant	0.645
Phenolics vs DPPH	0.980	Significant *	0.961	0.627	Non-significant	0.961
Phenolics vs FRAP	0.990	Significant**	0.974	0.694	Non-significant	0.974
Flavonoids vs Phenolics	0.803	Non-significant	0.645	0.514	Non-significant	0.645
Flavonoids vs DPPH	0.684	Non-significant	0.467	0.952	Significant**	0.906
Flavonoids vs FRAP	0.712	Non-significant	0.487	0.874	Non-significant	0.487
	<i>E. herbacea</i>			<i>E. epidendrea</i>		
Phenolics vs Flavonoids	0.696	Non-significant	0.484	0.886	Non-significant	0.784
Phenolics vs DPPH	0.820	Non-significant	0.671	0.993	Significant *	0.985
Phenolics vs FRAP	0.994	Significant*	0.988	0.977	Significant**	0.954
Flavonoids vs Phenolics	0.766	Non-significant	0.587	0.886	Non-significant	0.784

Flavonoids vs DPPH	0.913	Non-significant	0.832	0.900	Non-significant	0.809
Flavonoids vs FRAP	0.879	Non-significant	0.772	0.675	Non-significant	0.803
<i>E. ramentacea</i>			<i>E. andamanensis</i>			
Phenolics vs Flavonoids	-0.567	Non-Significant	0.321	0.781	Non-Significant	0.648
Phenolics vs DPPH	0.978	Significant **	0.955	0.845	Non-Significant	0.714
Phenolics vs FRAP	0.965	Significant**	0.916	0.982	Significant **	0.964
Flavonoids vs Phenolics	-0.567	Non-Significant	0.321	0.781	Significant **	0.648
Flavonoids vs DPPH	-0.642	Non-significant	0.412	0.741	Non-Significant	0.548
Flavonoids vs FRAP	0.834	Non-significant	0.695	0.947	Non-Significant	0.896

\*significant at  $\alpha = 0.01$ , \*\* significant at  $\alpha = 0.05$

In *E. ochreatea* the biochemical parameters showed poor correlation with DPPH, FRAP and phenolics, suggesting compounds other than these may involved in antioxidant activity. *E. herbacea* showed strong positive correlation between TPC and FRAP ( $R^2=0.994$ ) implying strong reducing potential of phenolic compounds contributing for free radical scavenging activity, while poor correlation in TPC, TFC and DPPH was observed. In *E. epidendrea* TPC was significantly correlated with DPPH and FRAP ( $R^2=0.993$  and  $R^2=0.977$  respectively).

In *E. ramentacea*, and *E. andamanensis* significant correlation was observed with total phenolics and its antioxidant activity as determined by FRAP and DPPH activity. In *E. ramentacea*, total flavonoid content showed negative correlation with phenolics and antioxidant activity, measured in terms of DPPH assay. However the same showed positive, non-significant correlation with FRAP.

The antioxidant activity of plant extracts is not limited to phenolics and flavonoids, but also includes vitamins C and E, carotenoids and chlorophylls [21]. It is also observed that the major antioxidant components might not be phenolics but it could be sterols, tocopherols, ascorbic acid and/or carotenoids [22].

High correlation coefficients between the phenolic content and antioxidant activities have been reported for various food commodities, for example, cactus pear ( $R^2=0.970$ ) [23]. Therefore, total phenolic content can be used as an indicator in assessing the antioxidant activity of fruits and vegetables. The Folin-Ciocalteu method for total phenolic determination can be standardized for comparison of the result between laboratories [24].

The present study in *Eulophia* species revealed that there is strong correlation in phenolics and antioxidant activity. The high degree of correlation between the simple spectrophotometric assay for total phenolic compounds and antioxidant activity as determined by radical quenching assays, shows that the assay for total phenolics would be a useful technique for rapid evaluation of antioxidant activity in *Eulophia* species. This would be important for breeding programs where many samples must be evaluated for antioxidant activity between genotypes.

#### 4. Acknowledgement

Authors are thankful to Department of Botany, Shivaji University, Kolhapur, Maharashtra, for providing necessary facilities to carry out the present work.

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