EVALUATION OF THE TOTAL ANTIOXIDANT CAPACITY OF THE SELECTED PLANT EXTRACTS USING FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

K. Manikya Kumari[*1] and V. Padmaja[2]

[1]*Senior Lecturer, Department of Botany, St. Joseph's College for Women (Autonomous), Visakhapatnam-530004, Andhra Pradesh, India*
[2]*Professor, Department of Botany, Andhra University, Visakhapatnam-530003, AP, India*
Corresponding author*: mk.kambham@gmail.com

Abstracts

Back ground: Antioxidants are compounds capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. The cumulative and synergistic activities of the bioactive molecules present in plants are responsible for their enhanced antioxidant properties. The present study is aimed at the *in vitro* evaluation of the Total antioxidant capacity of the plant extracts of *Kydia calycina* aerial parts, *Alysicarpus monilifer* whole plant and *Aglaia elaeagnoidea* stem.

Method: The FRAP method relies on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)- 1,3,5-triazine). The binding of Fe2+ to the ligand creates a very intense navy blue colour. The absorbance can be measured to test the amount of iron reduced and can be correlated with the amount of antioxidants and their total antioxidant capacity.

Result: From the results it was found that, among the hydro alcoholic extracts evaluated, *Kydia calycina* plant extract showed highest FRAP value (1.41) and *Alysicarpus monilifer* showed lowest value (0.88).

Conclusion: The total antioxidant potential is a relevant tool for investigating the relationship between plant derived antioxidants and pathologies induced by the oxidative stress. In the present investigation, comparative evaluation of Total antioxidant activity of various extracts of selected medicinal plants in comparison to Ascorbic acid exhibited significant results *in vitro* antioxidant assays.

Keywords: Antioxidants, Total antioxidant capacity, FRAP assay, FRAP Value, *Kydia calycina, Alysicarpus monilifer, Aglaia elaeagnoidea*

1. Introduction

There is an increasing interest in antioxidants, which have attracted considerable attention in relation to free radicals and oxidative stress, cancer prophylaxis and therapy, and longevity.[1] Phenols and polyphenols are the target analytes in many such cases; they may be detected by enzymes like tyrosinase or other phenol oxidases, or even by plant tissues containing these enzymes.[2-5] The role of free radicals has been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, aging , arthritix, diabetes, etc.[6] and the compounds that can scavenge free radicals have great potential in ameliorating these diseases process.

Antioxidants are compounds capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. They are used for the stabilization of polymeric products, of petrochemicals, foodstuffs, cosmetics and pharmaceuticals. Antioxidants are involved in the defence mechanism of the organism against the pathologies associated to the attack of free radicals.

Endogenous antioxidants are enzymes, like superoxide dismutase, catalase, glutathione peroxidase or nonenzymatic compounds, such as uric acid, bilirubin, albumin, metallothioneins. When endogenous factors cannot ensure a rigorous control and a complete protection of the organism against the reactive oxygen species, the need for exogenous antioxidants arises, as nutritional supplements or pharmaceutical products, which contain as active principle an antioxidant compound. Amongst the most important exogenous antioxidants, vitamin E, vitamin C, β-carotene, vitamin E, flavonoids, mineral Se are well known, but also vitamin D and vitamin K.

Exogenous antioxidants can derive from natural sources such as vitamins, flavonoids, anthocyanins, some mineral compounds, but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene, gallates, etc.[7]

The total antioxidant potential is a relevant tool for investigating the relationship between plant derived antioxidants and pathologies induced by the oxidative stress. Several studies on antioxidants proved to reduce risk of both cardiac and distal gastric cancer.[8] Several analytical methods were recently developed for measuring the total antioxidant capacity of therapeutic herbs, these assays differ in the mechanism of generation of different radical species and/or target molecules and in the way end-products are measured.[9-11]

Among components fighting against chronic diseases, great attention has been paid to phytochemicals, plant-derived molecules endowed with steady antioxidant power. The cumulative and synergistic activities of the bioactive molecules present in plants are responsible for their enhanced antioxidant properties. Hence, an appropriate investigation of the role of antioxidants from natural source such as plants in disease prevention is need of the hour.[12]

Ascorbic acid is an antioxidant with therapeutic properties, which plays an important role in activating the immune response, in wound healing, in osteogenesis, in detoxifying the organism, in iron absorption, in collagen biosynthesis, in preventing the clotting of blood vessels, and in many other metabolic processes.[13,14]

Special attention has been focused on the study of antioxidant action mechanism. The excess free radicals circulating in the body oxidize the low density lipoproteins (LDL), making them potentially lethal; the excess free radicals can also accelerate aging processes and have been linked to other very serious pathologies, such as brain stroke, diabetes mellitus, rheumatoid arthritis, Parkinson's disease, Alzheimer's disease and cancer. Physiologically, the oxygenated free radicals are among the most important radical species. Reactive oxygen species (ROS) comprise species with a strong oxidizing tendency, both of a radical nature, for example the superoxide radical, the hydroxyl radical and a non-radical nature eg,: ozone, hydrogen peroxide.[15]

Antioxidant actions might be exerted by inhibiting generation of reactive oxygen species and reactive nitrogen species, or by directly scavenging free radicals or by raising the levels of endogenous antioxidant enzymes by up regulating expression of the genes encoding superoxide dismutase, catalase of alutathione peroxidase.[16]

The present study is aimed at the *in vitro* evaluation of the 'Total antioxidant capacity' of the plant extracts of *Kydia calycina* aerial parts, *Alysicarpus monilifer* whole plant and *Aglaia elaeagnoidea* stem using FRAP method.

2. Materials and Methods

2.1 Reagents

i) 300mM Acetate buffer

10g of sodium acetate trihydrate was weighed and 16ml of glacial acetic acid was added and the final volume was made up to 1L using distilled deionised H_2O

ii) 40 mM HCl

3.4 ml of Conc. HCl was measured and made up to a final volume of 1L using distilled deionised H_2O.

The following reagents were prepared fresh on the day of the assay.

iii) 10mM TPTZ (2, 4, 6-tripyridyl- *s*- triazine)

123g TPTZ /1L 40 mM HCl

iv) 20mM $FeCl_3.6H_2O$

5.406g/L using distilled deionised H_2O

v) FRAP reagent

Standard curve: Acetate buffer: TPTZ: H_2O in the ratio of 10: 1: 1

Samples: Acetate buffer: TPTZ: $FeCl_3.6H_2O$ in the ratio of 10: 1: 1

vi) Standard

Cuvette method: 0.001M $FeSO_4 7H_2O$

2.78g of $FeSO_4 7H_2O$ was weighed.

The final volume was made up to 1L using distilled deionized H_2O.

1/10 dilution was made to obtain 0.001M concentration.

vii) Standard Curve

Table 1: The following reagents were added into glass tubes (add standard last)

	1	2	3	4	5	6	7	8
H_2O (µl)	1000	985	970	940	880	820	760	700
1mM Std(µl)	0	15	30	60	120	180	240	300
FRAP (ml)	3	3	3	3	3	3	3	3
Std Conc. (µM)	0	5	10	20	40	60	80	100

For Blank: 3ml FRAP Reagent + 1ml H_2O

Into a cuvette add 100µl sample + 900µl distilled H_2O + 3ml FRAP reagent

2.2 Standards and controls:

Aqueous solutions of known Fe (**II**) concentration ($FeSo_4$ $7H_2O$) and /or freshly prepared aqueous solutions of a pure antioxidant, such as ascorbic acid used for calibration of the FRAP assay. Reaction of Fe (**III**) represents a known electron exchange reaction and is taken as unity, i.e., the blank corrected signal given by 100µM solution of Fe(**II**) is equivalent to a FRAP value of 100µM. Typical Fe(II) standard concentration used in the laboratories are in the range of 100-1000µM. Ascorbic acid has a constant stoichiometric factor of 2.0 in the FRAP assay, i.e., direct reaction of Fe (II) gives a change in absorbance half that of an equivalent molar concentration of ascorbic acid. An ascorbic acid standard of 1000 µM, therefore, is equivalent to 2000µM of antioxidant power as FRAP. For standard curve, aqueous ascorbic acid solutions were prepared in the range from 100 to 1000 µM (equivalent to 200 to 2000µM FRAP) prepared freshly.

The FRAP (Ferric Reducing Antioxidant Power) method:

The FRAP method relies on the reduction by the antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)- 1,3,5-triazine). The binding of Fe2+ to the ligand creates a very intense navy blue colour. The absorbance can be measured to test the amount of iron reduced and can be correlated with the amount of antioxidants[17]Trolox or ascorbic acid[18] were used as references.

Determination of Ferric reducing or Antioxidant power assay (FRAP)

The total antioxidant power of the sample was measured by ferric reducing antioxidant power (FRAP) assay of Benzie IFF and JJ Strain (1996)[19]. The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method employing an easily reduced oxidant system present in stoichiometric excess.

Principle

At low p^H, deduction of a ferric tripyridyl triazine [Fe (III)-TPTZ] complex to the ferrous form, which has an intense blue color, can be monitored by measuring the change in absorption at 593nm.The reaction is non- specific in that any half-reaction that has a lower redox potential, under reaction conditions, than that of the ferric/ferrous half-reaction will drive the ferric (FeIII) to ferrous (FeII) reaction. The change in absorbance, therefore, is directly related to the combined or “total” reducing power of the electron donating anti oxidants present in the reaction mixture.

Procedure

This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the ferrous form at low pH of 3.6. This reduction is monitored by measuring the change in absorption at 593 nm, using a diode-array spectrophotometer.

3 ml of prepared FRAP reagent is mixed with 100 µL of diluted sample and 900µL of distilled H_2O. This solution is vortex mixed and incubated for 30min at 37 °C in the dark. The absorbance was read at 593nm against a reagent blank at a predetermined time after sample-reagent mixture. FRAP values can be obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe3+ and expressed as mM of Fe2+ equivalents per kg or per L of sample. The results are expressed as Ascorbic acid equivalents (µ moles/ml) or FRAP units.

Calculations

FRAP value of the sample (µM) =
Change in absorbance of sample from 0-4 minutes/ change in absorbance of standard from 0-4 minutes x FRAP value of standard (1000µM)

(or)

FRAP value of the sample (µM) =

$$\frac{\text{Abs (sample) x FRAP value of Std.}(\mu M)}{\text{Abs (Std.)}}$$

Note: FRAP value of Ascorbic acid = 2

Draw a standard curve using Absorbance Vs Fe^{2+} Conc. (X) and obtain equation for best fit line.

Use sample absorbance values that have been zeroed (i.e. Abs sample – Abs blank) to express FRAP in molarity.

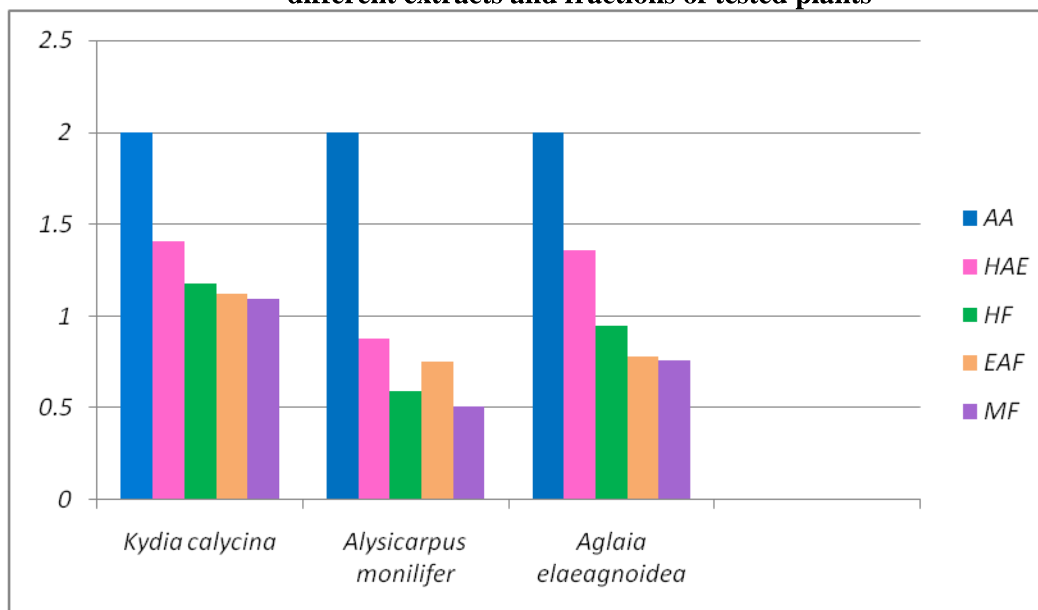
3. Results

From the results it was found that, among the hydro alcoholic extracts evaluated, *Kydia calycina* plant extract showed highest FRAP value (1.41) followed by *Aglaia elaeagnoidea* (1.36) and *Alysicarpus monilifer* showed lowest value (0.88). Among the fractions of the selected plants tested, hexane fraction of *Kydia calycina* showed highest FRAP value (1.18) followed by hexane fraction of *Aglaia elaeagnoidea* (0.95) and the methanol soluble fraction of *Alysicarpus monilifer* showed lowest value (0.51) as shown in Table 2 & Fig. 1.

Table: 2 FRAP values of Standard Ascorbic Acid extracts and fractions

Plant Name	Sample	FRAP value
	Ascorbic Acid	2.00
Kydia calycina	Hydro alcoholic extract	1.41
	Hexane fraction	1.18
	Ethyl acetate fraction	1.12
	Methanol fraction	1.09
Alyysicarpus monilifer	Hydro alcoholic extract	0.88
	Hexane fraction	0.59
	Ethyl acetate fraction	0.75
	Methanol fraction	0.51
Aglaia elaeagnoidea	Hydro alcoholic extract	1.36
	Hexane fraction	0.95
	Ethyl acetate fraction	0.78
	Methanol fraction	0.76

Figure 1: Total antioxidant activity determined by FRAP method showing FRAP values of different extracts and fractions of tested plants



AA: Ascorbic acid; HAE: Hydro-Alcoholic Extract; HF: Hexane Fraction; EAF: Ethyl Acetate Fraction; MF: Methanol Fraction

4. Discussion

FRAP assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. Total antioxidant power may be referred analogously to total reducing power. In the current study the antioxidant activity of the selected plant extracts of *Kydia calycina* aerial parts, *Alysicarpus monilifer* whole plant and *Aglaia elaeagnoidea* stem were evaluated.

The ferric reducing antioxidant power of a plant extract depends on its total phenolic content and bioactive phytoconstituents present in it. *Kydia calycina* revealed the presence of bioactive triterpene and flavones glycosides such as squalele, friedelin and tiliriside, they must have been responsible for the observed high FRAP value (1.41). Although *Alysicarpus monilifer* showed the presence of important bioactive phenolic compound like phytosterols and flavoniod glycosides namely vitexin and isivitexin, they might not have shown the activity in synergy, in spite of their performance as good antioxidants individually. Therefore *Alysicarpus monilifer* recorded low FRAP value (0.51) as shown in Table 2.

Natural antioxidants such as phenolic acids, flavonoids and tannins possess potent antioxidant activity.[20-21] Sterols like β – sitosterol has been reported to have anti – oxidant activity. The triterpinoids are also reported to possess anti oxidant activity.[22]

Qualitative phytochemical tests reveal that *K. calycina* contains phytosterols, triterpinoids, alkaloids, flavonoids, tannins and glycosides; *A. elaeagnoidea* contains sterols, terpinoids, alkaloids, flavonoids, tannins and glycosides*;* *A.monilifer* contains phytosterols, terpinoids, saponins, flavonoids tannins, carbohydrates and glycosides. These active constituents alone or in combination might be responsible for the observed anti oxidant activity in the present study.

Isolation and characterization of bioactive phytochemicals of the three selected plants revealed the presence of β-sitosterol, stigmasterol, lenoleic acid, squalin and tiliroside in the aerial parts of *Kydia calycina*; β-sitosterol, stigmasterol, ursolic acid, isovitexin, vitexin and inositol in the whole plant of *Alysicarpus monilifer*; β-sitosterol, stigmasterol, freidalenin, daucosterol and other unidentified polyphenolic compounds in the stem of *Aglaia elaeagnoidea* might be responsible for the observed antioxidant activity either singly or in synergy.

5. Conclusions

In the present investigation, comparative evaluation of Total antioxidant activity of various extracts of selected medicinal plants in comparison to Ascorbic acid exhibited significant results *in vitro* antioxidant assays. These antioxidant studies proved efficacy of extracts and fractions of the tested plants. The findings show that it is a safe and effective intervention for free radical mediated therapy using these evaluated medicinal plants.

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