REVIEW ARTICLE Am. J. PharmTech Res. 2012; 2(4) ISSN: 2249-3387

AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

A Perspective on Antioxidants

Ramakrishna T.M[1]., Mahuya De Ghosh[2]*, Ravikumar H.[1]

1. Dept. of Biological Science, Bangalore University, JB Campus Bangalore- 560 056. :

2. Dept of Research & PG Studies in Chemistry, Indian Academy, Degree College, Hennur Cross, Bangalore 560-043

ABSTRACT

The antioxidant capacity of foods, medicines and their supplements can be estimated by following procedures of certain methods involving spectrophotometer which is discussed here. The methods followed have been slightly modified sometimes, to suit the needs of botanicals. All the procedures, given here require spectrophotometer to asess the ability of absorbance. The significance of antioxidants in diets, medicines and nutraceuticals is enumerated. Each method has its own ability of reacting with either reductants or enzymes, or free radicals.Thus antioxidant capacity requires two or three methods to assess the capacity of antioxidants present in foods and medicines.

Keywords: significance of antioxidants, methods in assessment

*Corresponding Author Email: demahuya@yahoo.com

Received 19 June 2012, Accepted 29 June 2012

Please cite this article in press as: Ghosh MD *et al.*, A Perspective on Antioxidants. American Journal of PharmTech Research 2012.

INTRODUCTION

The free radical chain reactions occur in the body during the normal metabolic process and there by reactive oxygen species and reactive nitrogen species are generated. The ROS and RNS include diverse reactive entities namely superoxide (O_2) Hydroxyl ($OH^{.}$), Peroxy ($ROO^{.}$), Peroxinitrate ($ONOO^{.}$), the Nitric oxide ($NO^{.}$) radicals as well as non free radical species as Hydrogen peroxide (H_2O_2) Nitrous acid (HNO_2) and Hypochlorous acid (HOCl) , (Yildrin *et al* 2000, Gulcin *et al* 2002, Mavi *et al* 2003)[1-3]. These ROS and RNS are known to cause damage to lipids, proteins, enzymes, nucleic acids leading to cell or tissue injury. They are also implicated in the process of ageing as well as wide range of degenerative diseases, including inflammation, cancer, atherosclerosis, diabetes, alzheimers, parkinson's disease, coronary heart pathologies. The ROS and RNS role is pivotal for the modulation of critical cellular functions, such as apoptosis programmed activation and ion transport, calcium mobilization involved in excitotoxicity, especially in neurons, astrocytes and microglia. The excitotoxicity and apoptosis are the two main causes of neuronal death. The role of mitochondria especially its dysfunction, leading to the cell energy impairment. (Emerit *et al* 2004, Duan *et al* 2006)[4,5].

Now, oxidative stress is recognized as accountable for redox regulation involving ROS and RNS. The free radicals and ROS can be removed by natural antioxidant defense in the body, for example, glutathione peroxidase, catalase and superoxide dismutase (Aruoma 1994)[6]. However, overproduction of ROS arising from either by mitochondrial electron transport chain or excessive stimulation of NADPH or from exposure to environmental pollutants like cigarette smoke, UV rays and toxic chemicals (Valko *et al* 2006)[7] results in weakened body defense system.

The majority of antioxidant activity is due to the flavones, isoflavones, flavanols, anthocyanins, coumarins, lignans, catechins and isocatechins (Aquil 2006)[8]. The antioxidant based drug formulations are used for the prevention of treatment of various complex diseases like atherosclerosis stroke, diabetes, alzheimers and cancer parkinson's disease, coronary heart pathologies (Devasagayam *et al* 2004, Polterait 1997)[9,10]. To prevent the human body from oxidative stress due to ROS and RNS a defense mechanism is present. It may be of enzymatic and nonenzymatic. The enzymatic mechanism include for the existence of superoxide dismutase, catalase, glutathione reductase, peroxidase and nitric oxide synthase enzymes, among non enzymatic mechanisms comprises antioxidants and trapping agents. Among high mol. wt. compounds proteins like albumin, transferin, ceruplasmin are responsible to restrict the production of metal catalyzed free radicals (Khanam *et al* 2004, Chae *et al* 2004)[11,12]. Similarly, low mol. wt. Compounds include 2 types, lipid soluble and water soluble antioxidants. The tocopherol, qunines, bilirubin and some polyphenols are lipid soluble antioxidants and ascorbic acid, uric acid and some polyphenols are water soluble antioxidants (Blois 2004, Chae *et al* 2004)[13,12]. Minerals like, selenium, copper manganese, zinc etc are well known antioxidants. Chromium is also known to have antioxidant property. The vitamins such as vitamin A, C and E are said to be popular antioxidants which play a crucial role in peroxidation damage in the biological systems (Fogaliano *et al* 1999, Montena *et al* 2003)[14,15].

The potential source of antioxidants is plant. Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties and more so plants have unmatched chemical diversity. Plant extracts may be either pure compounds or standardized extracts and vegetables used in traditional medicine can provide diverse secondary metabolites with antioxidant potencies. Most of these are isolated phenolic compounds (Ramarathnam *et a*l 1997 Odukoya *et al* 2005)[16,17]. These species provide opportunities for new drug discoveries (Cos *et al* 2006)[18]. According to World Health Organization (WHO) more than 80% of the human population depends upon traditional medicine for their primary health care needs. This captured the interest of many researchers in the world to explore local medicinal plants for valuable medicinal traits. In addition the antifungal drugs with distinct modes of action need to be identified because of increasing incidence of fungal resistance to existing antibiotics (Loeffler and Stevans 2003)[19]. Majority of the plant secondary metabolites are antioxidant compounds which are potential source of effective and antifungal agents (Duarte *et al* 2004)[20]. Plant derived compounds such as hydroquinones and naphthaquinones (Lapachol, Juglone) sesquiterpenes (Cinnamodial, Capsidiol) and alkaloids such as berberine has shown antimicrobial and antifungal activities. An advantage of the approach of using ethnobotanical data is to identify compounds with antimicrobial activity. Many of the remedies have been used by traditional healers for themselves, for years with few or no adverse effects.

The natural antioxidant mechanism in mammals under certain circumstances can be inefficient, a dietary intake of antioxidant compounds becomes alternative, once it has been suggested that there is an inverse relationship between dietary intake of antioxidants and disease caused by the deficiency of these substances (Antolovich et al 2002)[21]. In these years, the synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) are added to food preparations because they are good free radical scavengers, even though there are some experimental proofs that, they induce DNA damage (Sasaki et al 2002)[22]. Therefore, thrust was given to find antioxidants from natural origin. (Goncalves *et al* 2005)[23].

A general concensus has been reached during the last few years that diet has a major role in the development of chronic diseases such as cancer, coronary heart disease, diabetes type 2, hypertension and cataract. Studies have shown that consumption of food and beverages rich in phenolic content is correlated with reduced incidence of heart diseases, anaemia, asthma, arthritis inflammation, and neurodegeneration.(American Institute of Cancer Research 1997[24], US Dept of Agriculture 1995[25], American Heart Association 1996[26], American Cancer Society 1996[27], World Health Organization 1990[28], Willet 1998, 1999[29,30], Jacob's *et al* 1998[31], US Dept of Health and Human Services 1996[32],(Mahuya and Ramakrishna 2011)[33]. In addition, there aroused a considerable interest in finding natural antioxidants from plant resources, which are considered as safe. The scientists were looking for natural antioxidants from dietary, cosmetic and pharmaceutical sources. There is a need to provide the body with constant supply of phytochemicals through dietary supplementation. These antioxidants have the ability to prevent, delay, ameliorate the effects of free radicals (Hargguchi 2001[34], Polterait 1997[10)].

MATERIALS AND METHODS:

The plant materials such as roots, stems, leaves, flowers, fruits and seeds are used separately to determine the expression of the antioxidant property. In case of Ayurvedic system of medicine (One of the oldest Indian systems of medicines), leaf, bark, stem, root, flowers are collected in particular part of the day like early morning before sunrise or in the evening, before the sunset. These parts are being used in drug preparations along with other combinations. These plant parts are separately air dried in shade. Among the dried plant materials, like seeds, are directly used in the estimation of antioxidants.

These plant materials are powdered and extracted using different solvents like petroleum ether, chloroform, ethyl acetate, methanol, ethanol, n-butanol using soxhlet apparatus. In addition to the above, cold water extract and hot water extract are also practised. The crude extracts were filtered and concentrated at reduced temperature using rotary evaporator.

Thiobarbituric Acid Test (TBA) Assay

TBA test was conducted by Mackeen *et al* (2000)[35], Ottolenghi (1959)[36]. This is as follows –

1.0 ml of 20% aqueous tricholoroacetic acid and 2.0ml of 0.67% aqueous thiobarbituric acid were added to 2ml of sample solutions is prepared in 1ml of ethanol solution of the extract(5mg/ml). The mixtures were then placed in boiling water bath for 10 minutes. After cooling under the running tap water, the mixtures were centrifuged at 3000g for 30 minutes. Finally, the absorbance of supernatants was measured at 532nm using spectrophotometer.

TEAC or other ABTS assay: (Trolox equivalent antioxidant capacity)

TEAC assay was reported by Miller *et al* (1993) [37] which is based on the scavenging ability of antioxidants, the long life radical anion $ABTS^{\cdot -}$. In the assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\cdot +}$, which is intensely coloured and assessed the antioxidant capacity. This is the ability of test compounds to decrease the colour reacting directly with $ABTS^{+}$ radical. The result of the test compounds are expressed relative to Trolox (Ronald *et al* 2005) [38]The absorption maxima of $ABTS^{\cdot +}$ were shown to be at wavelength of 415,645,734 and 815nm. Among them 415 and 734 were adopted by most of the investigators to spectrophotometrically monitor the reaction between the antioxidants and ABTS. + (Cano *et al* 2000)[39].

ABTS. + reacts rapidly with antioxidants, typically within 30 min. It can be used over a wide pH range and can be used to study effectively of pH on antioxidant mechanisms (Lemanska *et al* 2001)[40]. $ABT^{\cdot +}$ is not affected by ionic strength and it is soluble in both aqueous and organic solvents. This is the reason it can be used to determine both hydrophylic and lipophilic antioxidant capacities of extracts and body fluids (Awika *et al* 2003)[41]. Thermodynamically a compound can reduce ABTS. + if it has redox potential lower than that of $ABTS^{\cdot + (o.68v)}$. Many phenolic compounds have low redox potentials and can thus react with $ABTS^{\cdot +}$. The $ABTS^{\cdot +}$ radical used in TEAC assays is not found in mammalian biology and thus represents a 'non physiological' radical source (Ronald *et al* 2005)[38].The ABTS assay could also be done according to Re *et al* (1999)[42] with some modifications. The ABTS radical cation ($ABTS^{+}$) is produced by reacting 7m M stock solution of ABTS with 2.45 m M Potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 h before use. The $ABTS^{+}$ solution was diluted with n-butanol to an absorbance of 0.800 ± 0.05 at 734nm. Later 2.85 ml of $ABTS^{+}$ solution is added to 0.15 ml of different concentrations of the extracts and the decrease in absorbance at 734 nm is noted. The radical scavenging activity of the tested extract is also expressed with IC 50 value.

Scavenging activity of DPPH radical:

DPPH radical scavenging activity is performed according to Nenadis and Tsimidou (2002)[43]. DPPH in ethanol (250 1M, 2ml) was added to 2 ml of the test compounds at different concentrations in ethanol. The final concentrations of the test compounds in the reaction mixtures were 0.5,5, 10, 25, 50µM. Each mixture was shaken well vigorously and held in dark for 30 min. at room temperature. The decrease in absorbance of DPPH at 517nm was measured. Ethanol was used as blank solution .DPPH solution (2 ml) served as control. Generally, the tests were conducted in triplicate. The radical scavenging activity of the samples expressed as percentage of inhibition of DPPH absorbance.

Percentage of inhibition= (A control – A test) /A control $\times$ 100

Where A control is the absorbance of the control (DPPH solution without test sample) and A test is the absorbance of the test sample (DPPH solution plus compound). Ascorbic acid and Tocopherol were used as reference compounds. According to Erasto *et al* (2004)[44], radical scavenging ability of DPPH method is as follows. Methanolic solution (1 ml) of different extracts at 100 mg/ml concentration was added to 1 ml methanolic solution of DPPH (2mg/ml). The absorbance is measured at 517nm after 30 min. The results were evaluated as scavenging percentage of radical. (% of scavenging of DPPH = Abs of blank – Abs of sample/ Abs of blank × 100). The results are compared with standards (gallic acid and BHT) as mentioned in the other method.

The DPPH radical is one of the stable organic nitrogen radicals, which forms deep blue colour. It is commercially available and does not have to be generated before assay like $ABTS^+$ This assay is based on the measurement of reducing ability of antioxidants toward DPPH. The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. The decolouration assay was first reported by Brand – Williams and coworkers (1995)[45]. Antioxidant assays are based on the loss of DPPH colour at 515nm after reaction with test compound (Bondet *et al* 1997)[46]. The percentage of the remaining DPPH is calculated as

$$\% \ DPPH_{REM} = 100 \times (DPPH)_{REM} / (DPPH)\ T{=}0$$

The percentage of remaining DPPH ($DPPH_{REM}$) is proportional to the antioxidant concentration and concentration that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} is defined as TEC_{50}. Sanchez Moreno *et al* (1998)[47] introduced another parameter to express antioxidant capacity called "Antiradical Efficiency (AE)" It is defined as AE = $I/EC_{50}\ .TEC_{50}$. Efficient concentration (mg antioxidant/mg DPPH)

FRAP Assay:

Ferric reducing antioxidant power (FRAP) was originally developed by Benzie and Strain (1996)[48], but with modifications used for the assay of antioxidants in botanicals (Benzie and Szeto 1999)[49]. The reaction measures reduction of ferric 2,4,6 tripyridyl –s-triazine (TPTZ) to a coloured product. The FRAP reaction detects, compounds with redox potentials of < 0.7v (the redox potential of Fe^{3+}-TPTZ), so FRAP is a reasonable screen for the ability to maintain redox status in cells or tissues. Reducing power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols (Pulido *et al* 2000)[50]. The FRAP assay is done according to Benzie and Strain (1996)[48] with some modifications. The stock solution included 300 mM acetate buffer (pH 3.6), 10mM TPTZ solution in 40mM HCl and 20 mM $FeCl_3$ $6H_2$ O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5ml of TPTZ solution and 2.5 ml of Fe Cl_3 $6H_2$ O solution. The extracts (200ml) were allowed to react with 3800µl of FRAP solution for 30 min at 37^{o}C. Reading the colored product (ferrous tripyridyltriazine complex) was then measured at 593 nm. Trolox was used as a reference standard and results were expressed in µ mol, Trolox equivalents (TE)/g extracts. Additional dilution was needed if the FRAP value is measured was over the linear range of the standard curve.

Ferric thiocyanate method (FTC):

The antioxidant activity of different extracts on inhibition of linoleic acid oxidation was assayed by ferric thiocyanate (Osawa and Namiki 1981)[51].1 ml of the ethanolic solution of the extract (5mg/ml) was mixed with 10 ml of absolute ethanol, 10 ml of 0.2M phosphate buffer (pH 6.0) and of 2% (v/v) linoleic acid. All the samples were incubated at 40^{o}C. At regular intervals (48h), 5ml ethanol. 0.1 ml of 0.02M ferrous chloride in 3.5% HCl and 0.1 ml of aqueous 20% ammonium thiocyanate was added to the solution and absorbance was recorded a 500nm using spectrophotometer. Gallic acid or BHT or Tocopheral can be used as standard reference in 2 mg/ml concentration.

Folin-Ciocalteu (Total Assay of phenols, polyphenols):

In total antioxidant capacity assays –phenols, polyphenols plus reducing agents and also possibly of metal chelators is done. The F-C assay has for many years been used as a measure of total phenolics in natural products, but the basic mechanism is an oxidation/reduction reaction. The F-C method was developed in 1927 which originated from chemical reagents used for tyrosine analysis (Folin 1927)[52], in which oxidation of phenols by molybdotungstate reagent yields a coloured product and then spectrophotometric reading is taken at 745-750 nm.

Later Singleton and Rossi (1965)[53] improved the method with molybdotungsto-phosphoric heteropolyanion reagent

$3\ H_2O$ - P2 O_5 - 13 WO_3 - $5MoO_3$ - 10 H_2O and $3H_2O$ – P_2 O_5 - $14WO_3$ – $4MoO_3$ – 10 H_2 O

that reduced phenols more specifically and Spectrophotometric reading at 765 nm is taken. Singleton and Rossi imposed mandatory steps and conditions to obtain reliable and predictable data: 1) proper volume ratio of alkali and F-C reagent; 2) optimal reaction time and temperature for colour development; 3) monitoring of optical density at 765 nm; and 4) using gallic acid as the reference –standard phenol. Thus specified the conditions to minimize variability and eliminate erratic results.

The procedure for F-C method of Singleton and Rossi is as follows:

To 1 ml of sample (properly diluted) with at least 60ml of water, 5ml of F-C reagent is added, after 30sec and before 8min, 15ml of Na_2CO_3 solution is added and volume made upto 100ml with water, the same is incubated for 2hrs at 75°F and absorbance measured at 765nm. According to Singleton and Rossi (1965)[53] 'compared to permanganate oxidation or ultraviolet absorbance methods, this method produces predictable results from wide range of phenolics'. A slight variation in F-C method was followed by Makkar et al (1993)[54], to estimate the total phenolic contents of the extracts. The procedure is as follows:

0.1 ml of extract was combined with 2.8 ml 10% Na_2CO_3 and 0.1 ml of 2N Folin-Ciocalteu reagent. The absorbance was measured after 40 min at 725 nm using UV-visible spectrophotometer. The results were determined as mg equivalent of gallic acid per gram of extract.

CONCLUSION:

The challenge in the assay of antioxidant capacity within the biological systems is difficult because of several reaction characteristics and mechanisms involved. No single assay will reflect all the radical sources or all antioxidants in a mixed or complex system (Ronald *et al* 2005)[38]. There is no single simple method by which antioxidant capacity can be measured accurately and quantitatively. The methods given here are all based on spectrophotometric analysis. Two or three methods described here should be used to assess the antioxidants present in a system. The reactive oxygen species causing various pathophysiological and endogenous mechanisms have evolved to offer protection. Any increase in antioxidant consumption can reduce oxidative stress. The extracts of plants can be considered as new sources of natural antioxidants for food and nutraceutical products. The potential source of antioxidants is plants. Therefore, extensive knowledge of antioxidants from plant sources is required to estimate them accurately. The F-C method is simple, sensitive and precise. However, the reaction is slow at acidic pH and it lacks specificity.

REFERENCES:

1. Yildrin A, Mavi A, Oktay M, Kara A A, Algur O F, Bilaloglu V. Comparison of

antioxidant and antimicrobial activities of tilia (*Tilia argentea* Desf Ex DC) sage (*Salvia triloba* L.) and black tea (*Camelia sinensis*) extracts. J Agric Food Chem 2000; 48: 5030-5034

2. Gulcin I, Buyukokuroglu ME, Oktay M, Kufrevioglu OL. On the in vitro antioxidative properties of melatonin. J Pineal Res 2002;33(3):167-171
3. Mavi A, Terzi Z, Ozoen U, Yildrim A, Coskun M. Antioxidant properties of some medicinal plants: *Prangos ferulacea* (Apiaceae), *Sedum semipervivoides* (Crassulaceae) *Malva neglecta* (Malvaceae), *Cruciata turica* (Rubiaceae) *Rosa pimpinellifolia* (Rosaceae), *Gallium verum* sub sp *verum* (Rubiaceae) *Urtica dioica* (Urticaceae). Biol. Pharma. Bull 2003; 27:702-705.
4. Emerit J, Edeas M, Bricaire F. Neuro degenerative diseases and oxidative stress. Biomed. Pharmacother 2004 58:39-46.
5. Duan XJ, Zhang WW, Lix M, Wang BG. Evaluation of antioxidant property of extract and fractions obtained from red alga Polysiphonia urceolata. Food Chem 2006; 95; 37-43.
6. Auroma OI. Nutrition and health aspects of free radicals and antioxidants. Food Chem. Toxic 1994; 32:677-683.
7. Valko M, Leibfritz D, Moncol J. Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease Int J Biochem Cell Biol 2006; 7(10):45-78.
8. Aquil F, Ahmed I, Mehmood Z. Antioxidant and free radical and antioxidants in human health. Curr Stat fut Pros Jap 2006; 53:794-804.
9. Devasagyam TPA, Tilak JC, Boloor KK. Review; Free radical antioxidants in Human health. Curr Stat Fut Pros Jap 2004;55: 286-301
10. (a) Hargguchi, H. Antioxidative plant constituents. In Bioactive compounds from Natural sources, first publ. New York: Taylor and Fracis Inc 2001:338-377

10. (b)Polterait, O. Antioxidants and radical scavengers of Natural origin Current Org. Chem.1997; 1; 415-440.

11. Khanam S. Shivaprasad, HN, Devi K. In-vitro antioxidant screening models: a Review Indian J. Pharm. Edu. 2004; 38(4):181:1199-1200.
12. Chae S, Kim JS, Kang KA. Buhd Lee Y, Hyum JW. Kang. Antioxidant activity of Jionoside D from *Clerodendron trichotomum*. Biol Pharm Bull 2004; 27:1504-1508.
13. Blois M. Antioxidant determinations by the use of a stable free radical Nature 2004; 181:1199-1200.

14. Fogliano V, Verde V, Randazzo G, Ritieni, A. Method for measuring antioxidant activity and its application in monitoring the antioxidant capacity of wines. J Agric Food Chem1999; 47:1035-1040.
15. Montena SK, Jagadish, Badduri SR, Siripurapu KB, Unnikrishnan MK. In vitro evaluation of antioxidant properties of *Cocos nucifera* Linn. Water. Nahrung Food 2003; 2:126-131.
16. Ramarathnam, N. Ochi H. Tekeuchi M. Antioxidants defense system in vegetable extracts in: Shadi F(ed,) Natural antioxidants: Chemistry, Health effects and Applications. Aca Press Champaign, IL1997:70-87.
17. Odukoya OA, LLori OO, Sofidiya MO, Aniunon OA, Lawal BM, Tade IO. Antioxidant activity of Nigerian dietary spices. Electr J Environ Agric Food Chem 2005; 4(6):1086.
18. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger *in vitro* proof-of-concept. J Ethnopharmacol. 2006; 106:290.
19. Loeffler J, Stevans DA. Antifungal drug resistance. Clin Infect Dis 2003 36(suppl.1):S31-S41.
20. Durate, Rocha, A. Braga de Oliviera, A. Dias de Souza, Filho, J. Lombardi, J.A. Castro Brag, F. Antifungal constituents of *Clystostoma remeataceum* and *Monsoa hirsute.* Phytother Res. 2004; 18:463-467.
21. Antolovich M, Prenzler P, Patsalides E. methods for testing antioxidant activity. Analyst 2002; 1237:183-198.
22. Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwamak Taniguchi K. Tsuda S. The Comment assay with 8 mouse organs: results with 39 currently used food additives. Mut Res-Gen Tox En 2002; 519:103-109.
23. Goncalves C. Dinis, T. Batista, M.T Antioxidant properties of Proanthocyanadins of *Uncaria tomentosa* bark decoction: a mechanism for anti-inflammatory activity. Phytochemistry 2005; 66:89-98.
24. World Cancer Research Fund/American Institute for Cancer Research Food, Nutrition and the Prevention of Cancer: A global Perspective American Institute for Cancer Research Washington, DC.1997.
25. U. S. Department of Agriculture, U. S. Department of Health, Human Services Nutrition and Your Health: Dietary Guidelines for Americans US Government Printing Office Washington, DC.1995.

26. American Heart Association Dietary guidelines for healthy American adults. Circulation 1996;94:1795-1800,
27. American Cancer Society Advisory committee on Diet, Nutrition and Cancer Prevention. Guidelines on diet, nutrition and cancer prevention reducing the risk of cancer with healthy food choices and physical activity. CA. Cancer J Clin 1996; 46:325-341.
28. World Health Organization Diet, Nutrition and the Prevention of Chronic Diseases: Report of a WHO Study Group–Technical Report Series 797, World Switzerland1990.
29. Willett WC. Goals for nutrition in the year 2000. CA. Cancer J Clin 1999;49:331-352,
30. Willet WC. Nutritional Epidemiology Oxford University Press New York, NY 1998.
31. Jacobs DRJ, Marquart L, Slavin J, Kushi LH. Whole- grain intake and cancer: an expanded review and meta-analysis. Nutr Cancer 1998; 30: 85-96, 31.
32. U.S. Department of Health and Human Services Physical Activity and Health: A Report of the Surgeon General U.S. Government Printing Office Atlanta, GA; 1996.
33. Mahuya De Ghosh and Ramakrishna TM. Antioxidant survey in certain food plants. Int J Pharm Bio Sci 2011 :1(2): 1-7
34. Hargguchi,H. Antioxidative plant constituents in bioactive compounds from Natural sources, first publ. Newyork: Taylor and Fracis Inc. 2001: 338-377
35. Osawa, T. Namiki, M. Antioxidative activity of three herbal water extracts. Agric Biol Chem 1981; 45:735-739.
36. Mackeen MM, Ali AM, Lagis NH, Hawazu K, Hassan Z, Amran M. Antimicrobial antioxidant, antitumor-promoting and cytotoxic activites of different plant parts extracts of *Garcinia atroviridin* Grifex T. Anders J. Ethnopharmacology 2000; 72:395-402.
37. Ottolenghi A. Interaction of ascorbic acid and Mitochondria lipids. Arch Biochem Biophy 1959:79: 355-363.
38. Miller NJ, Diplock AT, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method of measuring antioxidant capacity and its application to monitoring the antioxidant Clin Sci 1993; 84:407-412.
39. Ronald, LP. Xianli, Wu and Karen Schaich. Standardized methods for the determination of antioxidants capacity and phenolics in foods and dietary supplements. J Agric Food Chem 2005; 53:4290-4302
40. Cano A, Acosta M, Arnao MB. A method to measure antioxidant activity in organic media: application to lipophilic vitamins. Redox report 5: 365-370.

41. Re R, Pellogrini N, Proteggente A, Panala A, Yang M. and Rice-Evans C. Antioxidant activity applying in improved abts radical cation decoloration assay. Free Radic Biol Med 1999; 26:1231-1237.
42. Lemanska K, Szymusiak H, Tyrakowska B, Zielinski R, Soffer AEMF, Reietjeins, IMCM. The influence of pH on the antioxidant properties and the mechanisms of antioxidant action of hydroflavones Free Radical Biol Med 31:869-881.
43. Awika JM. Rooney LW, Wu X, Prior RL, Cisneros-Zevallos L. Screening methods to measure antioxidant activity of *Sorghum* (*Sorghum bicolor*) and *Sorghum* products. J Agric Food Chem 2003; 51:6657-6662.
44. Nenadis, M, Tsimidou. Observation on the estimation of scavenging activity of ph nolic compounds using rapid 1, 1-diphenyl-2-picrylhydrazyl (DPPH) tests. J Am Oilchem Soc 2002; 79:1191-1195.
45. Erasto P. Bojase-Moleta G. Majinda RRT. Antimicrobial and antioxidant flavanoids from the root wood of Bolusanthus speciosus Phytochemistry 2004;65:875-880.
46. Brand-williams W, Cuvelie ME, Berset C. Use of a free radical method to evaluate antioxidant asctivity. Lebensm Wiss Technol 1995; 28:25-30.
47. Bondet V, Brand-Williams W, Berset C. Kinetics and mechanisms of antioxidant activity using the DPPH free radical method Lebensm Wiss Technol 1997; 30:609-615.
48. Sannchez Mereno C, Larrauri JA, Saura-Calixto F. A procedure to measure the antiradical effiency of polyphenols. J Sci Food Agric 1996; 76:270-276.
49. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant Power": the FRAP assay. Anal Biochem 1996:111-116.
50. Benzie IIF, Szeto YT. Total antioxidant capacity of teas by the ferric reducing /antioxidant power assay. J Agric Food Chem 1999; 47: 633-636.
51. Pulido R, Bravo L, Saura-Calixto F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agric Food Chem 2000; 48:3396-3402.
52. Folin, O. Tyrosine and tryphtophan determinations in proteins. J Biol Chem 1927; 649.
53. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungistic acid reagents. Am J Enol Vitic119656: 144-158.
54. Makkar H, Blummel M, Borowy NK. Becker, K. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J Sci Food Agric 1993; 61:161-165.