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VISUAL ESTIMATION AND SPECTROPHOTOMETRIC DETERMINATION OF TANNIN CONTENT AND ANTIOXIDANT ACTIVITY OF THREE COMMON VEGETABLE

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ABSTRACT

Antioxidant activities of different vegetables Bitter gourd, Tomato and Spinach *(Momordica charantia, Lycopersicon esculentum, Spinacia oleracea)* were determined. In addition, rapid visual estimation of tannin content, total phenolic, tested for their free radical scavenging activity (FRSA) in the DPPH (1, 1-dephenyl- 2-picrylhydrazil radical) of those samples were assessed. A new technique was used to quickly distinguish between intermediate, moderately high and high tannin content by the development of shades of purple, light blue and deep blue colors (Bitter gourd, Tomato and Spinach) respectively. The total phenolics in methanol solution for all the samples were analyzed with slightly higher value for spinach paste. The % of condensed tannin of all the three vegetables were low as determined by the Vanillin- HCl method respectively. The antioxidant activity with DPPH- method expressed as per percent inhibition of oxidation ranged from a high of 88% in bitter gourd extract to near about 70% in tomato and spinach respectively. The studies can be further extended to exploit their possible application for the preservation of food products as well as health supplements and neutraceuticals.

INTRODUCTION: Antioxidants are defense against free radical and oxidative attacks. They act as free radical scavengers and slow down not only oxidation of radical but also the accompanying damaging effects in the body. Fruits and vegetables have conferred on them the status of functional foods [1]. They seem to be capable of delivering health benefits besides fulfilling physiological needs. Scientific evidence suggests that antioxidants reduce risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Routine or habitual consumption of fruits and vegetables confers significant benefits to human health [2].

Interest in the role of antioxidants in human health has prompted research in the fields of Bioinorganic, Food science and Horticulture to assess fruit and vegetable for their antioxidant properties.[3] The majority of the antioxidant capacity of a fruit or vegetable may be from compounds such as flavonoids, isoflavones, flavones, anthocyanin, ployphen, catechins and isocatechins rather than from vitamins C, E, β-carotene [4, 5], phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. In addition, some supplements, such as Zinc, Copper and Selenium, are necessary to actually strengthen the body's own antioxidant protection system [6].

Transition metal ions are powerful catalysts of oxidative reaction *in-vivo*. Iron is the most likely candidate for promoting oxidative reactions [7]. The release of 'free' metal ions (i.e., the release of metal ions in forms able to catalyze oxidative reactions) from sequestered sites can occur as a result of tissue injury by disease, trauma, toxins, and other causes [7]. There is strong evidence that, under pathological condition such as cataracts [8], atherosclerotic lesions and diabetes metals ions are released and can be detected in their free and harmful form in this work. Epidemiological data as well as *in-vitro* studies strongly suggest that food containing phytochemicals with anti-oxidation potential have strong protective effects against major disease risks including various cancers, cardiovascular diseases, [9-12] neurological diseases [3], rheumatoid arthritis and to improve immune system. However, the scientific under standing on identification, characterization and their physiological functions are not complete.

Tannins are phenolic compounds that precipitate protein. They are composed of a very diverse group of oligomers and polymers. The presence and consequent interaction of tannins are proteins in the seeds of cereals and legumes have been believed to be of the factors involved in reduced protein digestibility. Most berries, such as cranberries and blueberries contain both hydrolysable and blueberries contain both hydrolysable and condensed tannins.

Ferric ion (Fe^{3+}) is reduced to ferrous ion (Fe^{2+}) by tannin and other phenolics to form a ferric-cyanide ferrous ion color complex commonly known as Prussian blue respectively. Vanillin test is used in this work for quantification of condensed tannin. The Vanillin - HCl method is based on the ability of condensed tannin to react with vanillin in the presence of minerals acid to produce a red color. And this test is specific for flavon 3- olsdihydrochalcones and proantho cyanidins.

The analytical method measures the antioxidants activities using free radical scavenging activity are fast, easy and simple. DPPH is widely used to test the ability of compounds to act as free radicals scavengers or hydrogen donors and to evaluate antioxidant activity of foods [13]. The various methods used to measure antioxidant activity of food products can

give varying result depending on the specificity of the free radical being used as a reactant. Mostly the current focus is on the anti oxidant activity of flavonoids and phenolics are mainly [14] because of their redox properties which allow them to act as reducing agents, hydrogen donors, Singlet oxygen quenchers and metal chelators [15]. Their antioxidant activity is generally based on the number and location of the superoxide anion radical (O_2), the peroxyl radical (ROO) or the hydroxyl radical (OH) present as well as the presence of a 2-3 double bond and 4-oxofunction [16, 17].

Elimination of synthetic anti-oxidants in food application has given more impetus to exploring natural sources of anti-oxidants. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant components, but applies to the overall antioxidants capacity of the sample. A measure of the total antioxidant capacity will helps us understand the functional properties of foods. India is bestowed with diverse climatic condition conducive for the growth of different vegetable known for their nutritional values. So for, there has not been a study on anti-oxidant activity of vegetables grown and consumed here.

The main objective of this study was to screen a large number of vegetable consumed in the Indian diet with respect to their total phenolic content and anti-oxidant activities. In the present study the visual estimation of tannin contents, total phenolics and the antioxidant activity of some vegetables Bitter gourd, Tomato and Spinach *(Momordica charantia, Lycopersicon esculentum, Spinacia oleracea)* was evaluated.

EXPERIMENTAL:

Materials: All solvents/chemical used were analytical/BDR grade. DPPH was obtained from Merch, Mumbai, India. Vegetable were purchased from local market.

Preparation of extract: Prior to extraction [14] and analysis all vegetable were washed and subjected to size reduction using a knife and blended with a kitchen mixer to get a thick paste. 250 mg of plant paste sample was extracted with 10 ml of 100% methanol / 70% aqueous acetone and left it overnight. Filtered with Whatmann filter paper and make up the volume up to 25 ml will 100% methanol / 70% aqueous acetone.

Total Solids: The solids contents were gravimetrically determined by drying a 5.0gm aliquot in a vacuum over at 70^0C to constant weight.

UV-Visual spectrophotometer: UV Visual measurements were performed on a UV visible 1700 spectrophotometer SHIMADZU.

1- Visual Estimation of Tannin Contents:

Reagent:

(a) 0.5M $FeCl_3$ was prepared in 0.1N HCl

(b) 0.008M $K_3Fe(CN)_6$ prepared in 100 ml distal water.

Sample extract in a 2 ml measuring spoon was added to a 125 ml flask containing 50 ml of water. Contents were swirled frequently for 3 minutes. 1 ml aliquots from each flask were mixed with 1 ml of 0.5M $FeCl_3$ in 0.1N HCl followed by 1 ml of 0.008M $K_3Fe(CN)_3$. All volume were measured with Pasture pipette calibrated to a 1 ml volume color developed immediately, but observation were recorded after 1min to allow color to become more stable [18].

2. Determination of Total Phenolic Contents:
Prussian Blue Method:
Reagent:

(a) 0.5 M $FeCl_3$ was prepared in 0.1 N HCl

(b) 0.008 M $K_3Fe(CN)_6$ prepared in 100 ml distal water.

Take 0.1 ml of aliquot was taken. It was diluted within 60 ml of distilled water and added 3 ml of 0.5M $FeCl_3$ in 0.1N HCl and 3 ml of 0.008 M $K_3Fe(CN)_6$. Color develops immediately after 10-15 min.

Spectrophotometric measurement: The optical density of the above solution was determined at 725 nm. The phenolic content can be calculated by using the following formula [19]:

$$\% \text{ Phenolic content} = \frac{\text{OD x Factor x 25 x 100}}{\text{Conc. x wt. of sample (mg)}}$$

Tannic acid was used as the standard, and the total polyphenol content was expressed as tannic acid equivalent (mg/ml).

To Determine the Factor Value in Standard Tannic Acid Solution by Spectrophotometric Measurement: Spectrophotometer is used for the determination of optical density the 3 ml of 0.05M $FeCl_3$ was added to the 0.01, 0.02, 0.03, 0.04, 0.06, 0.08, 0.1, 0.12 ml of tannic acid followed immediately by timed addition for 3 ml of 0.008M $K_3Fe(CN)_6$, but omitting the extract and using distilled water is different volume used (Final volume in 60 ml). And now record the absorbance at 725nm wavelength in spectrophotometer of the different solution.

3. Determination of Condensed Tannin:
Vanillin - HCl Method:
Regent:

1. 4% Vanillin in methanol
2. 8% HCl in methanol
3. 70% aqueous acetone

1 ml of aliquot was added in 5 ml of 4% vanillin + 8% HCl solution and left for 5-10 min.

Spectrophotometric measurement: A blank of identical composition but omitting the extract was analyzed and subtracted from all other reading. The condensed Tannin can be calculated[20] by using the following formula:

$$\% \text{ CT} = \frac{\text{OD x Factor x 25 x 100}}{\text{Conc. x wt. of sample (mg)}}$$

4. Evaluation of the Free Radical Scavenging Activity (FRSA) in the 1, 1- diphenyl- 2-picrylhydrazyl (DPPH) Method: Radical scavenging activity (RSA) of the extracts was determined by DPPH method [20-25]. By different concentration of vegetables extract in 0.010, 0.025, 0.05, 0.10, 0.25, 0.50 ml (equivalent to 10, 25, 50, 100, 250 and 500 ppm) were taken in different test tube.

The total volume was adjusted to 1.0ml with MeOH; 4ml of 0.1mM methanolic DPPH was added to these tubes and shaken well[22]. The mixture was allowed to stand at room temperature for 20 min. The blank was prepared as above without any sample or standard. The changes in absorbance of the sample were measured at 520 nm. Radical scavenging activity (RSA) was expressed as the inhibition percentage and was calculated using the formula:

$$\text{RSA\%} = \frac{\text{Blank OD} - \text{Sample OD}}{\text{Blank OD}} \times 100$$

Where,
Blank OD = Absorbance of blank
Sample OD = Absorbance of sample

RESULT AND DISCUSSION:

Data expression: All results were obtained from a minimum of four independent experiment and the relevant means were calculated. Data were expressed on a dry weight basis. The result of antioxidant analysis of three commonly consumed vegetable in Oct-15th Nov. 2009 was 6.5, 6.2, and 5.5%. The bitter gourd extract was higher than tomato and spinach extract. Bitter gourd extract showed presence of higher amount of phenolics corresponding to tomato and spinach vegetables respectively.

Visual Estimation of Tannin Content: This test is based on the reduction by tannin and other polyphenols of ferric ion to ferrous ion, followed by formation of a ferricyanide- ferrous ion complex. The colored product (commonly know as Prussian blue) absorbs maximally at 720 nm. Initial the solution is yellow, the color of the reagent. Increasing amount of tannin resulted in the production of increasing amounts of the blue pigment, which absorbs the red end of the spectrum. The solution however, appears green because the blue end of the spectrum is still masked by unreacted ferricyanide. If the initial ferricyanide concentration is sufficiently low, it will become noticeably depleted with higher amounts of tannin. The result is a deepening of the green color, followed by a change to blue color. It should be emphasized that because these color vary with condition used, they only reflect relative tannin contents. However, once these precautions are taken vegetable can be ranked as high, moderately high and intermediate in tannin with reasonable certainty in **Table 1**. It shows the result of a typical determination tannin contents for three vegetable, by the visual estimation [18]. The color obtained the relative ranking of the vegetable according to tannin content presented.

TABLE 1: VISUAL ESTIMATION OF TANNIN CONTENT OF VEGETABLES

Sample	Total Solid %	Colour	Rank	Tannin-contents
Bitter guard	6.5±0.50	Purple	3	Intermediate
Tomato	6.2±0.20	Light blue	2	Moderately high
Spinach	5.5 ± 0.08	Deep blue	1	High

It rapid completion of the analysis is more crucial than precision, reagent can be added directly to the vegetables. Within a few second intermediate, moderately high and high tannin varieties give shades of purple, light blue and deep blue color.

The main problem with this approach is that the amount of extracted tannin is changing rapidly during the first minute or two. After a few minutes even low tannin varieties become deep blue. Intermediate, moderately high and high tannin vegetable can be easily distinguished in this way after a few minutes. Changes in color within several minutes are most pronounced for the visual estimation method. In table 1, tomato becomes light blue probably because it has a moderately high tannin contents than the bitter guard vegetables, which developed purple color due to intermediate tannin content. Spinach was deep blue and clearly identifiable as high in tannin content respectively. It means a low value by the visual estimation method is nearly certain to mean the vegetable a low in tannin, and a high value that it is high in tannin.

TABLE 2: TO DETERMINE THE PRUSSIAN BLUE METHOD AND VANILLIN-HCL METHOD BY SPECTROPHOTOMETRIC MEASUREMENT

Sample	Standard Spectrophotometric method			
	Prussian blue method (in methanol)		Vanillin HCl method	
	O.D.	Total phenolics %	O.D.	Condensed tannin %
Bitter gourd	0.030	0.68	0.018	0.405
Tomato	0.026	0.58	0.040	0.900
Spinach	0.087	1.96	0.013	0.293

Factor value in tannic acid (in mg/ml) = 0.225

The result of $FeCl_3/K_3Fe(CN)_6$ (Prussian blue) system provides a sensitive method for quantitative determination [19, 26-28] of dilute concentration of polyphenolics in vegetables solution. To determine the factor value in tannic acid was standardized by using Prussian- blue method [19]. The sensitivity of the test towards flavonoid compounds is sufficient to determine concentration less than 10^{-4}M.

The phenolic compounds present are 0.68%, 0.058% and 1.96% for bitter gourd, tomato and spinach respectively. By these methods [26-28] spinach showed the highest polyphenolic content as compared to the other two vegetables. The selective extraction of antioxidants from natural sources by appropriate solvent is very important in obtaining fraction with high AA, which can be used more efficiently at lower concentration to prevent the oxidative deterioration as occurs in food system. The prescribed method for the Vanillin-HCl method [20] is based on the amount condensed tannin. The several vegetables had different tannin content. The result of the present work to find percentage of condensed tannin bitter gourd, tomato and spinach had 0.405%, 0.900% and 0.293% respectively. The low value of condensed tannin present in spinach vegetables indicates better antioxidant activity.

Free Radical Scavenging Activity by DPPH method: Free radical scavenging potential of vegetable extract at different concentrations was tested by DPPH method [22, 27, 28] and the results are presented in **fig. 1** respectively.

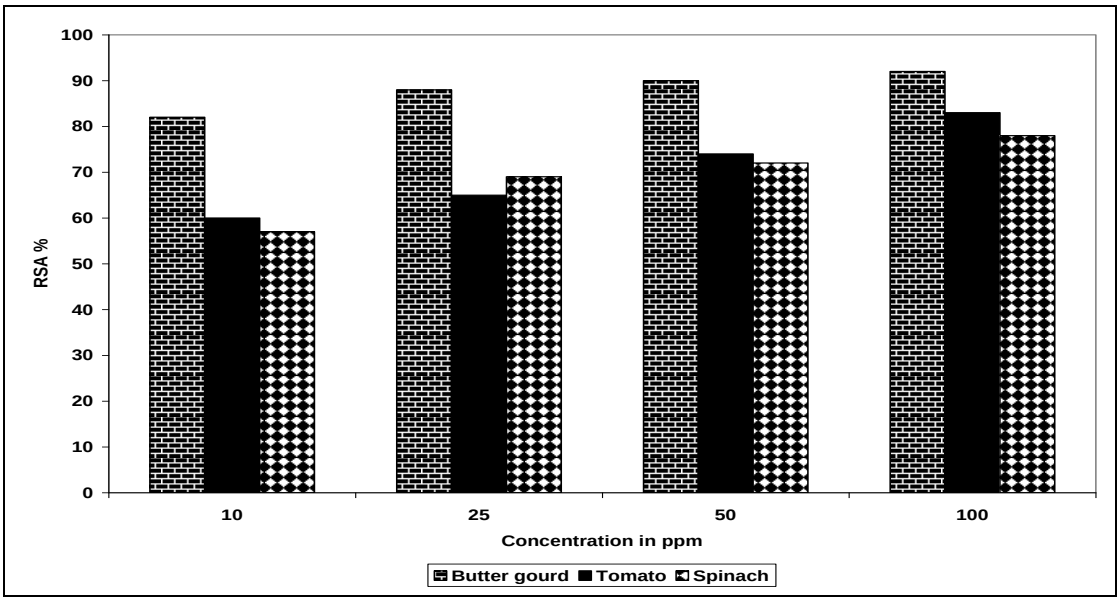

FIG. 1: DOES RESPONSE OF RADICAL SCAVENGING ACTIVITY (RSA %) OF BITTER GUARD, TOMATO AND SPINACH AT DIFFERENT CONCENTRATION BY DPPH METHOD

It can be seen that the different vegetables exhibition varying degrees of antioxidant capacity [21]. The average antioxidant of butter gourd, tomato and spinach were 88%, 70.5% and 69.5% RSA with methanolic DPPH solution respectively.

In this method, the reducing power of various extract is directly reflected by the absorbance value AA of each vegetable extract is proportional to the increase in the absorbance of the reaction mixture. The essence of DPPH method is that the antioxidants react with DPPH (1, 1- diphenyl- 2- picryl hydrazyl) and convert it to 1, 1- diphenyl- 2- picryl hydrazine with discoloration. The degree of discoloration at 520 nm indicates the scavenging potential of the antioxidant as has been used as a measure of AA.

The reducing properties are generally associated with the presence of reductones [29] whose antioxidant action is based on breaking of the free radical chain by donating one hydrogen atom [30]. Reductones also react with certain precursor of peroxide, thus preventing peroxide formation. The data indicate that the AA of bitter guard extracts may be due to presence of polyphenol, which may act similar to reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction.

The results of the present work indicate the presence of compound in vegetable possessing high anti-oxidant activity.

CONCLUSION: In the present study, a new analytical procedure can be used to provide a rapid and convenient estimation of the quality of anti-oxidant in the given vegetables. The visual estimation method is nearly certain to mean the vegetable a high value that it is high in tannin. The AA of various foods can be determined accurately, conveniently, and rapidly using DPPH. The study clearly indicates that it is important to measure the anti-oxidant activity using various radicals and oxidation system and to take both phenolic content and anti-oxidant activity into account while evaluating the anti-oxidant potential of vegetable extracts. The screened vegetables will be used for quality control purpose in industry. The health benefits from potential plant source and an additional information an their dietary intake and enhancing bioavailability will be proved.

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