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PHYTOCHEMICAL ESTIMATION AND ANTIOXIDANT ACTIVITY OF SEED EXTRACT OF MILLETS TRADITIONALLY CONSUMED BY COMMON PEOPLE OF UTTRAKHAND, INDIA

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ABSTRACT

Millets are small seeded grasses, highly nutritious and most digestible grains available in the world. These crops contain biologically active compounds which has been linked with potential antioxidant activities. Thus, the present study deals with the estimation of total phenol and flavonoid contents with the emphasis for determination of antioxidant activities of these millet's seeds. Phytochemical studies showed that protein, amino acid, carbohydrates, flavonoids, tannins, phenolics and tritenpenoids were present whereas alkaloids and glycosides were absent in all millet samples. The phenol concentration of millet samples range from 07.395±0.52 to 34.65±0.03. The highest phenolic concentration was found in the sorghum millet seed extracts (34.65 mg GAE/g extract). The flavonoid concentration of millet's samples varies from 16.53±0.29 to 47.55±3.98. The highest flavonoid concentration was found in the barnyard millet seed extracts (47.55 mg QE/g extract). The study was also conducted with reference to the reducing power activity which was highest in Italian millet as compared to standard(ascorbic acid).The antioxidant activity of five millet samples was calculated by DPPH scavenging activity. The IC_{50} value of millet samples ranged from 240-495µg/ml. The highest IC_{50} (450) value was obtained in Italain millet and lowest value (240) was obtained in little millet. These findings suggest that millet cultivation should be promoted as a food crop as they are rich in phenolic and flavonoids which are a good source of natural antioxidants.

Keywords: Millets, Antioxidant activity, Phenol, Flavonoids

INTRODUCTION

Free radicals and reactive oxygen species are well known inducers of molecular, cellular and tissue pathogenesis leading to several threats to the human society such as atherosclerosis, arthritis, cardiovascular diseases, ischemia, central nervous system injury, gastritis, cancer, aging and AIDS **[1, 2, 3]**. Not only the lack of effective therapies and oxidative damage plays a decisive etiological factor in many chronic conditions, the expediency of antioxidants in protection against these diseases is defensible. Antioxidants, agents that prevented the consumption of molecular oxygen in biological systems have multiple purposes, including defending against oxidative damage and participating in the major signaling pathways of the cells. Currently, the natural antioxidant and some synthetic antioxidants are commonly used to act against free radicals in food and biological systems. However, the use of synthetic antioxidants in food products is under strict regulation owing to their potential health hazards, low solubility **[4, 5]** and there is a trend to substitute them with naturally occurring antioxidants. Some natural antioxidants have been used commercially as nutritional supplements or, additives for their role in preventing human diseases. Also many other plant species have been investigated in the search for novel antioxidants **[6, 7, 8, 9]** but generally there is still a demand to find more information concerning the antioxidant potential of plant species.

Plants contain numerous primary and secondary metabolites. Several secondary metabolites such as polyphenols and flavonoids appreciated as chemo preventive agents are natural occurring substances and the most abundant antioxidants found in foods such as fruits, wine, tea, and cereals grains **[10-16]**. Cereals are the staple diet of most of the world's population. Millets are small seeded grasses, one of the oldest foods known to humans. They are highly nutritious and most digestible grains available in the world. In East Asia these crops are cultivated for emergency purposes and widely consumed due to their ability for nutrients deficiencies in rice, as in vitamins and minerals **[17, 18, 19**]. Beside this, these crops also contain biologically active compounds including tannins, phenols, anthocyanins, flavonoids which has been linked with potential antioxidant activities.

Therefore, the present study comprised of collection of seeds of five different millets from different localities of Uttarakhand followed by ethanol extraction for phytochemical analysis. Estimation of total phenol and flavonoid contents with the

emphasis for determination of antioxidant activities of these millet seeds.

MATERIAL AND METHODS

Plant Materials

The materials for the present study comprised of seeds of five different millets i.e. barnyard millet, little millet, sorghum millet, pearl millet and Italian millet collected from different localities of Dehradun, Uttarakhand. The present investigation was carried out at Biotechnology laboratory in Department of Life Sciences, Sri Guru Ram Rai Institute of Technology and Science, Patel Nagar, Dehradun, Uttarakhand. The millet samples were then crushed to coarse powder using grinder. The dried millet seeds materials were stored in paper bags.

Extraction

About 100 gram of the dried plant material powder was soaked with 70% ethanol for 24 hours at room temperature. Next day, the contents were refluxed for 2 hrs at temperature not exceeding 60^0C, cooled and filtered. Filtrate was evaporated to dryness at reduced temperature. The extract were stored in desiccators and used for further study.

Phytochemical Analysis

The ethanol extracts of seed samples of different millets were subjected to preliminary qualitative phytochemical investigation. The various tests performed were given below:

Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965) **[20]** with some modifications. 0.1 ml of sample and 50µl of 2 N Folin-Ciocalteu reagents was added to a 5 ml volumetric flask. The solution was mixed and allowed to stand for 3-5 min at room temperature. Next, 0.3 ml of 20% sodium carbonate solution (w/v) was added, and the solution was mixed and kept aside for 15 min. Finally, 5 ml of distilled water was added. The blue colour was measured against reagent blank at 725 nm using a UV-spectrophotometer. The total phenolic content of the sample was determined by comparison with the optical density values of different concentrations of the standard phenolic compound gallic acid. Each sample was analyzed in triplicate, and a calibration curve of gallic acid was constructed by plotting absorbance versus concentration. The total phenolic content was expressed as gram of gallic acid equivalents (GAE) per 100 gm extract.

Determination of Total Flavonoid Content

The total flavonoid content was determined with aluminum chloride ($AlCl_3$) method using quercetin as a standard. The extract

(0.25 ml each) was mixed with 1.25 ml double distilled water which was followed by the addition of 75µl of 5% $NaNO_2$. This mixture was incubated for 5 min at room temperature and then 0.15 ml of 10% $AlCl_3$ was added. The reaction mixture was treated with 0.5 ml of 1 mM NaOH. After an incubation of 6 min at room temperature. Finally, the reaction mixture was diluted with 5 ml of double distilled water followed by an incubation of 20 min at room temperature. The absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin standard curve. The total flavonoid content was expressed in milligrams of quercetin equivalents (QE) per gram of samples.

Determination of Reducing Power Activity

The reducing power of the sample was determined by the Oyaizu (1986) **[21]** method with some modification. Reducing power activity is based on the reduction of ferric cyanide (Fe^{3+}) in stoichiometric excess relative to the amount of antioxidants **[22]**. Sample (50µl) with different concentrations were mixed with 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 ml of 1% potassium ferric cyanide (w/v) and incubated at 50^{o}C for 20 min. After incubation, 2 ml of 10% trichloroacetic acid (w/v) were added to the mixture, followed by centrifugation at 30,000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2 ml of deionized water and 0.5 ml of 0.1% ferric chloride (w/v), and the absorbance of the resultant solution was measured at 700 nm. Ascorbic acid was used as Reference.

DPPH Radical Scavenging Assay

The scavenging activity was measured using 2, 2-diphenyl-1-picryl hydrazyl (DPPH). The millet extracts were redissolved in 70% ethanol. The 5 ml assay mixture contained 3.98 ml methanol, 20µl extract (50µl, 100µl, 150µl, 200µl), and 1 ml DPPH (0.15 mM in methanol). After incubation at room temperature for 30 min, the decrease in absorbance was measured at 517 nm using a spectrophotometer. Ascorbic acid was used as references. The IC50 value indicates the concentration of tested sample required to reduce the free radical concentration by 50%. The experiment was performed in triplicate.

DPPH Scavenging Activity (%) = $(A_0-A_1)/A_0\times100$

RESULT AND DISCUSSION

Millets are small seeded grasses, highly nutritious and most digestible grains available in the world. Beside this, these crops also contain biologically active compounds including tannins, phenols, anthocyanins, flavonoids which has been linked with potential antioxidant activities. The antioxidant activity of polyphenols is considered to be due to their properties as free radical terminators. This activity

depends mainly on different structural features such as O-H bound dissociation energy, resonance delocalization of the phenol radical and steric hindrance derived from hydrogen substitution in the aromatic ring **[23]**. The effect of polyphenols antioxidants on DPPH free radical scavenging is thought to be primary due to their hydrogen donating ability owing to the presence of hydroxyl groups **[24]**. The non-phenolic part of the molecule appears also to influence the activity of the conjugates, particularly their capacity to penetrate biological membranes, as well as the layers of the skin **[25]**. Thus flavan-3-ol conjugates with thiols have been showed to exhibit a higher antiradical capacity than their counterparts in the DPPH **[3, 14, 15]** suggesting the favorable role played by the non-phenolic part of the molecules in the antioxidant activity. Therefore, the present study comprised of collection of seeds of five different millets from different localities of Uttarakhand followed by ethanol extraction for phytochemical analysis. Estimation of total phenol and flavonoid contents with the emphasis for determination of antioxidant activities of these Millets seeds. The findings of the present study are as follows:

Yield and Preliminary Phytochemical Screening

Ethanol extract of millet seeds obtained by simple filtration method showed the variation in colour appearance ranges from light yellow (barnyard millet) to dark yellow (Italian millet) in **Table 1**. The percentage yield of crude extract ranged from 19-64%. The highest yield was 64% in sorghum millet and lowest yield are 19% in barnyard millet. Phytochemical screening of millets showed the presence of protein, amino acid, carbohydrates, flavonoids, tannins, phenolics and tritenpenoids and alkaloids in all the millet samples **(Table 2)**. Glycosides were not present in all millet samples.

Total Phenolic and Flavonoid Concentration

Phenolics are one major class of phytonutrients that have been widely studied, thus they are well known antioxidants compounds that work in multiple ways to prevent disease **[12]**. In the present study, the content of phenolics was evaluated from the regression equation of the calibration curve ($y= 0.070x + 0.078$, $R2=0.994$), expressed in GAE as milligrams per gram of extract (mg GAE/g extract) **(Figure 1)**. The total phenolic content of the five millet samples showed large variations ranges from 7.39±0.52 mg GAE/g to 34.65±0.03 mg GAE/g **(Table 3)**. The highest amount of phenol was found in the millet seed extract of sorghum millet (34.65±0.03 mg GAE/g extract) and lowest

amount of phenol was found in the seed extract of pearl millet (7.39±0.52mg GAE/g) whereas Issoufou *et al.*, (2011) reported that total phenolic content of different defatted foxtail millet bran extract varied significantly ($p<0.05$) among the extractions and ranged from 29.39±1.36 to 21.49±3.26 GAE/100 g extract **[26]**.

Flavonoids are well-known antioxidant constituents of plants and possess a broad spectrum of chemical and biological activity, including radical scavenging properties **[27]**. Therefore, the total content of flavonoids was evaluated from the calibration curve ($y = 0.084x-0.011$, $R^2 = 0.967$) expressed in QE as milligrams per gram of extract (mg QE/g extract) **(Figure 2)**. The content of flavonoids varied from 16.53-47.55 mg QE/g extract **(Table 3)**. The highest amount of flavonoids was found in the barnyard millet (47.55±3.98mg QE/g) seed extracts and lowest amount of flavonoids was found in the Italian millet (16.53±0.29mg QE/g) seed extracts.

Antioxidant Activity

Reducing Power Activity of Different Millets Samples

The reducing power activity i.e. the reduction of the $Fe^{3+/}$ferric cyanide of different millet extracts were determined from distinct colour changes (i.e. from yellow to green and blue) at 700nm, depending on the reducing power of the sample concentration. The high absorbance of the reaction mixture indicates high reducing power **(Figure 3)**. The study showed that highest reducing power activity was revealed by Italian millet and lowest reducing power was Barnyard millet as compared to standard (ascorbic acid).

DPPH Radical Scavenging Assay

DPPH scavenging activity of different millets were compared with standard (ascorbic acid) by evaluating antioxidant efficiencies, known as IC_{50}. The IC_{50} was the concentration of an antioxidant at which 50% inhibition of free radical activity was observed. The lower the IC_{50} number, the greater the overall effectiveness of the antioxidant in millet sample. The IC_{50} µg/ml ranged from 240-495µg/ml. IC_{50} value of barnyard millet (495µg/ml), Italian millet (395µg/ml), and sorghum millet (365µg/ml), pearl millet (350µg/ml) higher than standard (ascorbic acid) and lowest little millet (240µg/ml) **(Table 4)**. The % inhibition of different millet's samples and standard were compared with standard (ascorbic acid) (**Figure 4**). The highest value of % inhibition were shown by barnyard millet and lowest value were of little millet.

Table 1: Appearance and Yield of Crude Extracts From Ethanol Solvent of Different Millets Samples

S. No.	Samples	Appearance of the extract	Quantity of seed material(gm)	Weight of extract (gm)	Percentage yield
1.	Barnyard millet	Light yellow	100	0.19	19
2.	Little millet	Medium yellow	100	0.29	29
3.	Sorghum millet	Medium yellow	100	0.64	64
4.	Pearl millet	Dark yellow	100	0.51	51
5.	Italian millet	Dark yellow	100	0.47	47

Table 2: Phytochemical Analysis of Different Millet's Samples

S. No.	Constituents	Barnyard millet	Little millet	Sorghum millet	Pearl millet	Italian millet
1.	Alkaloids	-	-	-	-	-
2.	Proteins	+	+	+	+	+
3.	Amino acid	+	+	+	+	+
4.	Carbohydrates	+	+	+	+	+
5.	Flavonoids	+	+	+	+	+
6.	Glycosides	-	-	-	-	-
7.	Tannins & phenolics	+	+	+	+	+
8.	Triterpenoids	+	+	+	+	+

*** (+) and (-) signs indicate presence and absence of the compound, respectively**

Table 3: Total Phenol and Flavonoid Concentration of Different Millet's Samples

S. N.	Plant	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)
1.	Barnyard millet	10.125±0.03	47.55±3.98
2.	Little millet	12.33±1.29	44.84±1.52
3.	Sorghum millet	34.65±0.03	32.53±1.26
4.	Pearl millet	07.395±0.52	38.61±0.62
5.	Italian millet	28.87±1.03	16.53±0.29

Table 4: DPPH Assay IC_{50} values of different millet samples

S. N.	Plant	DPPH Assay IC 50(µg/ml)
1.	Barnyard millet	495
2.	Little millet	240
3.	Sorghum millet	365
4.	Pearl millet	350
5.	Italian millet	395
6.	Standard(Ascorbic acid)	300

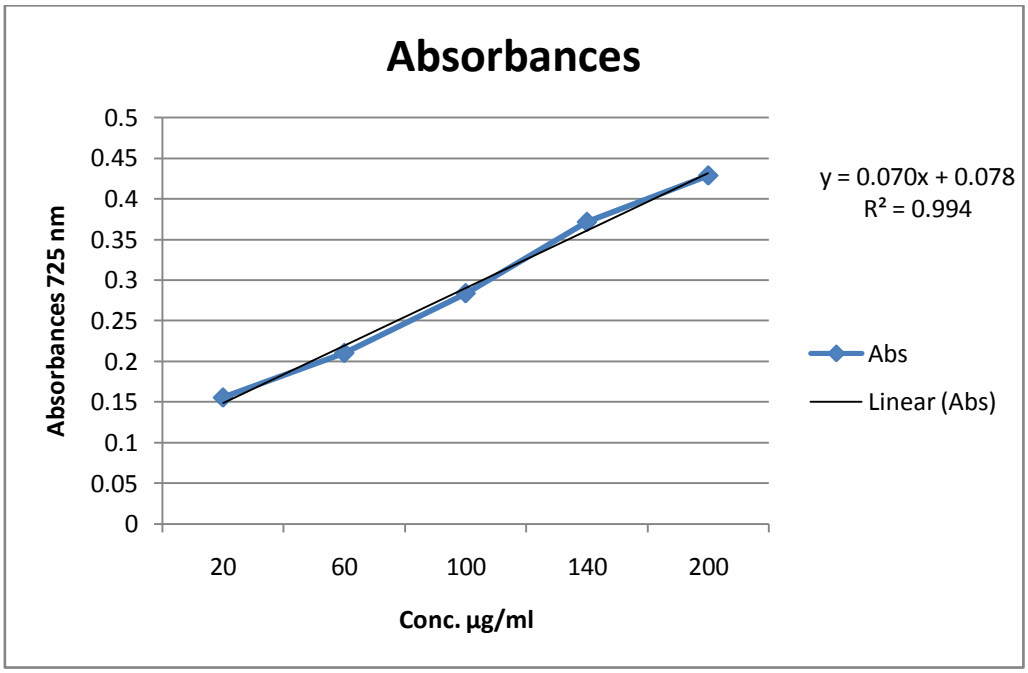

Figure 1: Standard Curve Represent Concentration of Gallic Acid (µg/ml) Against Absorbance

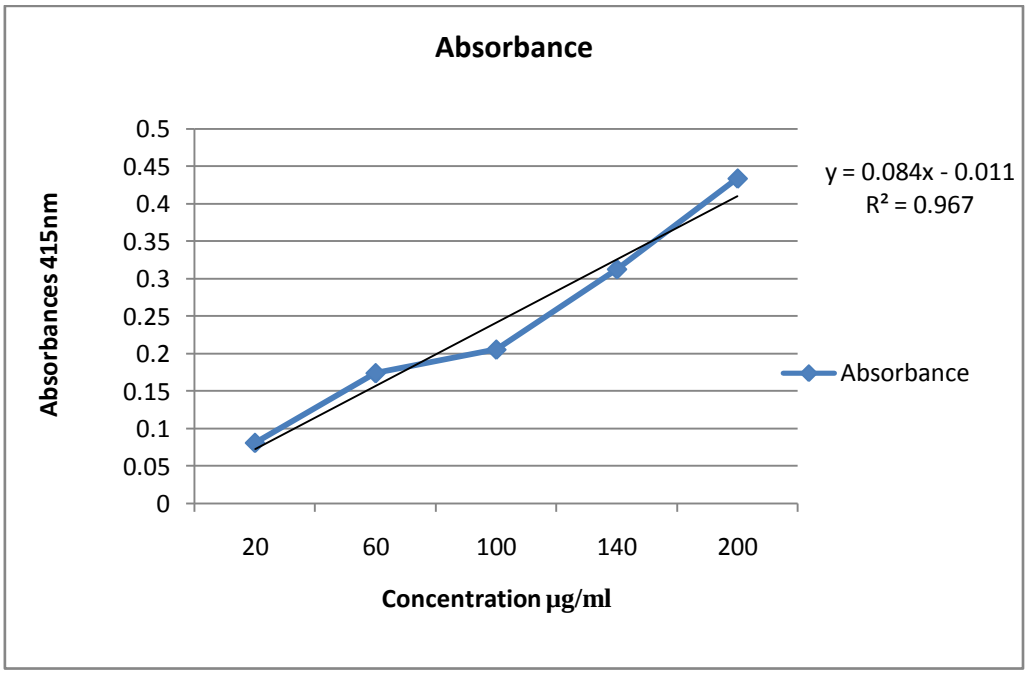

Figure 2: Standard Curve Represent Concentration of Quercetin (µg/ml) Against Absorbances

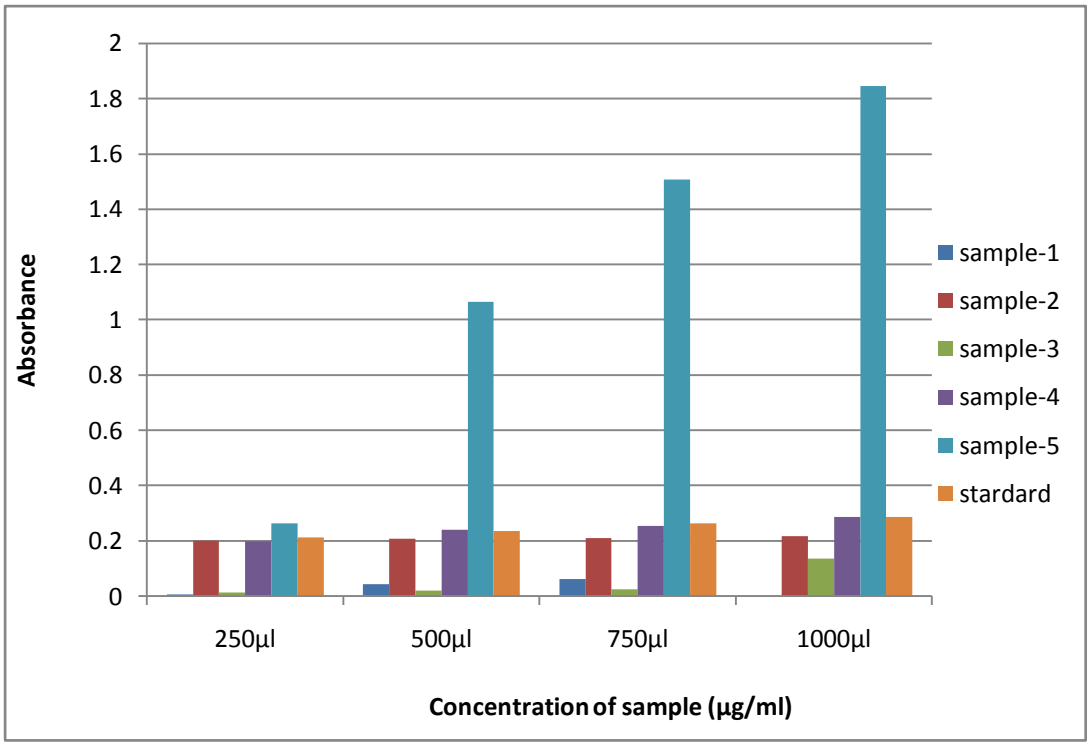

Figure 3: Reducing Power Compare of Different Millet's Samples

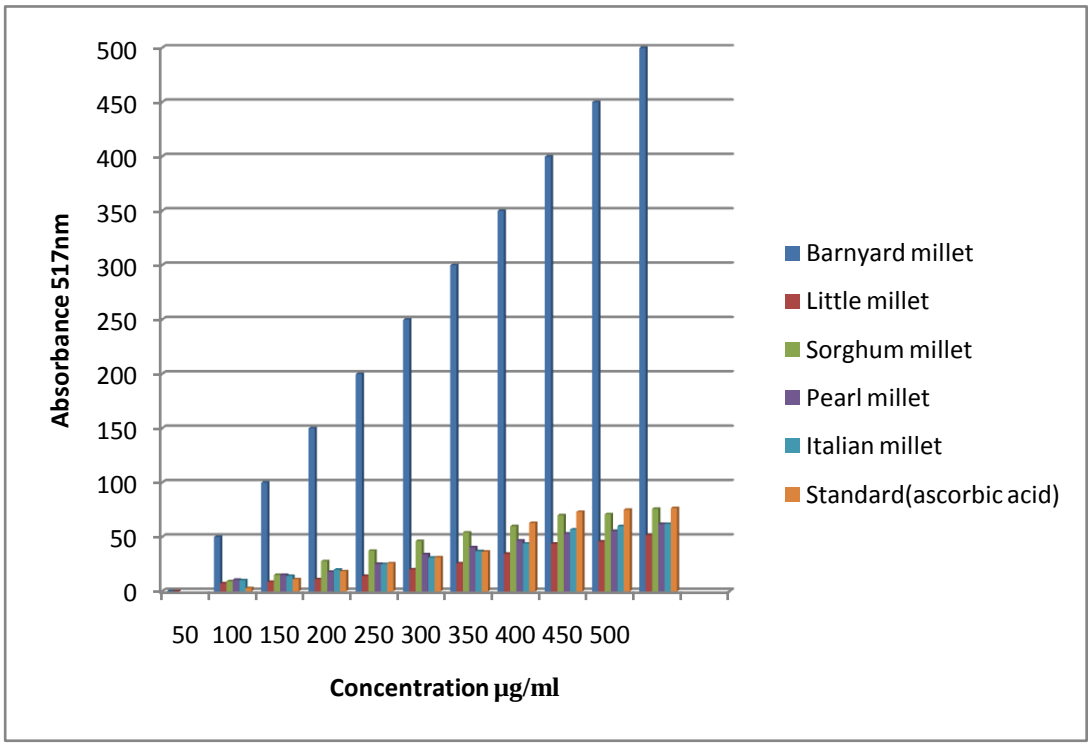

Figure 4: % Inhibition Compare of Different Millet's Samples and Standard

CONCLUSION

As millets are small seeded grasses, highly nutritious and most digestible grains available in the world containing biologically active compounds including tannins, phenols, anthocyanins, flavonoids which has been linked with potential antioxidant activities. Estimation of total phenol and flavonoid contents with the emphasis for determination of antioxidant activities of these millets seeds. Phytochemical studies showed that protein, amino acid, carbohydrates, flavonoids, tannins, phenolics and triterpenoids were present whereas alkaloids and glycosides were absent in all millet's samples. The highest phenolic concentration was found in the sorghum millet seed extracts (34.65 mg GAE/g extract), whereas the highest flavonoid concentration was found in the barnyard millet seed extracts (47.55 mg QE/g extract). The study was also conducted with reference to the reducing power activity with the highest IC_{50} (450) value which was highest in Italian millet and lowest value (240) was obtained in little millet. This study indicated that ethanol extracts of different millets possessed potent antioxidant activities, including reducing power, scavenging abilities on DPPH.These findings suggest that millet cultivation should be promoted as a food crop as they are rich in phenolic and flavonoids which are a good source of natural antioxidants. Further investigations on the relationship between phenolic and non phenolic content and antioxidant activity of different millet extracts were needed.

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