



In vitro Antioxidant and Phytochemical Analysis of Aqueous and Kamadhenu Ark Extracts of Nutricereals Millets

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ABSTRACT

Background: Globally the usage of Ethnomedicinal herbs to treat various diseases and metabolic disorders has increased over recent years. Intensive efforts are carried out by researchers in the last five decades to identify the phytochemicals responsible for Antibacterial, Antiviral, Antifungal, Antimicrobial, Antibiotic, Anticancer, Antidiabetic, Antihypertensive and Antioxidant Properties. Millets are a group of small-seeded grasses. Although millets are frequently referred to as coarse cereals, they are now known as nutricereals because of their greater levels of nutrients. The present study aims at the phytochemical analysis and evaluation of antioxidant potential of aqueous and kamadhenu ark extracts of few minor millets namely proso, little and kodo millets.

Methods: Proso, little and kodo millets are washed, shade dried and homogenized. The Aqueous and kamadhenu ark extracts were prepared by maceration method. Antioxidant activity was determined using DPPH Assay. Phytochemical Analysis were investigated as per standard procedures.

Result: Kamadhenu ark extract of proso millet has the highest antioxidant activity with IC₅₀ value of 0.0212±0.04 mg/ml and the least activity of 3.1952±0.19 mg/ml is shown by sprouted kodo millet in combination with kamadhenu ark. The Phytochemical analysis manifested the inherence of carbohydrates, diterpenes, glycosides, phenols, phytosterols, saponins, steroids, tannins and triterpenoids.

Key words: Antioxidant, Ethnomedicinal herbs, Kamadhenu ark, Millets, Nutricereals, Phytochemical analysis.

INTRODUCTION

Medicinal plants are nature's blessings to Mankind to heal numerous diseases. Globally the usage of Ethnomedicinal herbs to treat various diseases and metabolic disorders has increased over recent years (Archana *et al.*, 2012). A wide number of Indian medicinal plants are used consistently as antibiotics by clinicians of the Ayurveda and Unani systems of medicine and intensive efforts are carried out by researchers in the last five decades to identify the phytochemicals (Mayba *et al.*, 2019) responsible for antibacterial, antiviral, antifungal, anticancer, antidiabetic, antihypertensive and antioxidant properties Mohan *et al.* (2021).

The chemical process which involves transfer of electrons is oxidation. Free radicals are produced during oxidation reactions (Marak *et al.*, 2019). Free radicals trigger cascade of reactions which are harmful. Antioxidants eliminates the intermediary radicals produced during oxidation processes and stops the cascade of reactions. Antioxidants, which are generally reducing substances like thiols, ascorbic acid, or polyphenols, do this by getting oxidised themselves. All the living organisms possess sophisticated process containing various antioxidants, like vitamin C and E, glutathione, enzymes and various peroxidases. Inadequate antioxidants or suppression of antioxidant enzymes may attribute to oxidative stress which may lead to cell death. The pathophysiology of a various human ailments, including Parkinson's, Alzheimer's disease, neurodegenerative illnesses, diabetes, cancer and rheumatoid arthritis are intertwined with oxidative stress (Pawle and Singh, 2014). It also plays a role in a variety of

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renal disorders, including acute renal failure, obstructive nephropathy, hyperlipidaemia and glomerular injury. In acute renal failure patients receiving haemodialysis and urolithiasis, studies demonstrate that with higher lipid peroxidation and decline in antioxidative protection oxidative stress is elevated (Perumal Swamy *et al.*, 2008).

Plant bioactive elements called phytochemicals enhance health and are primarily generated by plants to preserve them. To genetically create designer foodstuffs, herbal preparations and beverages, these phytochemicals are isolated as nutrients, dietary supplements and specialized diets. Some of the phytochemicals usually we analyse are Flavonoids, phytoestrogens, polyphenols,

anthocyanidins, fibre, terpenoids, carotenoid, phytosterols and glycosides (Murugan *et al.*, 2022; Moatasem *et al.*, 2023).

Millets are a group of small-seeded grasses, commonly farmed as cereals for fodder and human nourishment all over the world (Handique *et al.*, 2023). Millets are major crops in Asia and Africa's semiarid tropics particularly in India and Nigeria (Chauhan *et al.*, 2018). Millets may have been utilized by humans since seven thousand years. Millets played a "critical role in the establishment of multi-crop agriculture and permanent farming civilizations," according to the researchers (Vanathi *et al.*, 2023). Sorghum (Great millet), Bajra (Pearl millet), Ragi (Finger millet) and small millets such as Korra (Foxtail millet), Little millet, Kodo millet, Proso millet and Barnyard millet are all important millet crops cultivated in India (Malathi *et al.*, 2016). Although millets are frequently referred to as coarse cereals, they are now known as nutricereals because of their greater levels of nutrients. Himanshu *et al.* (2018).

Although Millets are referred as *Nutricereals*, (Desai, and Dutta, 2023) the phytochemicals and Antioxidant Properties are unexplored. The Present Study aims at the phytochemical analysis and evaluation of Antioxidant activity of Aqueous and Kamadhenu Ark extracts (Ipsita Mohanty *et al.*, 2014) in combination with few millets namely proso, little and kodo millet.

MATERIALS AND METHODS

Preparation of extracts

Proso Millet (*Panicum miliaceum*), Kodo Millet (*Paspalum scrobiculatum*) and Little Millet (*Panicum sumatrense*) were washed, shade dried for one day and homogenized for the preparation of raw millet extracts. The Sprouted Millets extracts were prepared by soaking the sample in distilled water for 12 hours, later it was tied in muslin cloth and allowed it to germinate for 12 hours, then the sample was dried under room temperature for one day and homogenized to obtain Aqueous Proso Millet (APM), Aqueous Sprouted Proso Millet (ASPM), Aqueous Kodo Millet (AKM), Aqueous Sprouted Kodo Millet (ASKM), Aqueous Little Millet (ALM) and Aqueous Sprouted Little Millet (ASLM).

The early morning void urine of Kapila Cow (certified as healthy by veterinarian) was collected from the goshala for preparation of Kamadhenu Ark Extracts namely Kamadhenu Ark Proso Millet (KPM), Kamadhenu Ark Sprouted Proso Millet (KSPM), Kamadhenu Ark Kodo Millet (KKM), Kamadhenu Ark Sprouted Kodo Millet (KSKM), Kamadhenu Ark Little Millet (KLM) and Kamadhenu Ark Sprouted Little Millet (KSLM), The Aqueous and Kamadhenu Ark Extracts were prepared by Maceration method (Karagoz *et al.*, 2015).

Preparation of aqueous and kamadhenu ark extracts are carried out in DST-FIST, Department of Applied Microbiology and Biochemistry, Sri Padmavati Mahila Visvavidyalayam, Tirupati andhra Pradesh, from March 2019 to February 2020.

Phytochemical analysis

Aqueous extracts and kamadhenu ark extract of raw and sprouted proso, kodo and little millets were subjected to phytochemical analysis. Phytochemical screening for carbohydrates is done by using molisch test, for glycosides using legal test, for amino acids using ninhydrin test, for proteins using biuret test, for fixed oils and fats using spot test, for flavonoid's using test with sodium hydroxide, for phenols and tannins using neutral ferric chloride test, for alkaloids wagner's test, for saponins foam test, for steroids, phytosterols and triterpenoids using salkowski test, for coumarins test with alcoholic sodium hydroxide and for quinones test with aqueous sodium hydroxide (Murugan *et al.*, 2022).

Determination of total phenolic content

Using a 24-well microplate method, total phenolic content (TPC) was determined as per Ainsworth and Gillespie's protocols with a few small tweaks. Precisely, 100 micro litre sample extracts, standard (gallic acid) and blank (95% (v/v) methanol) dissolved in 400 µl double distilled water, mixed thoroughly with a quick spin. 150 µl of Folin-Ciocalteu reagent was added and vortexed. After five minutes, 500 µl of 20% Na₂CO₃ solution was added, vortexed and incubated for sixty minutes in dark. The absorbance was measured at 650 nm with imark Microplate Reader. The gallic acid standard curve was adopted to calculate the total phenolic content, which was then represented as mg of gallic acid equivalents (GAE) per g of sample (Ofoso *et al.*, 2020).

Evaluation of total terpenoids

Evaluation of total terpenoids was done using the protocol stated by (Indumathi *et al.*, 2014). 100 mg (W₁) of sample is soaked in 9ml of ethanol for 24 hours. The contents were filtered and extracted and the weight W₂ noted. Total terpenoids contents percentage of yield was measured by the formula:

$$\frac{W_1 - W_2}{W_1} \times 100$$

(Malik *et al.*, 2017).

Evaluation of total saponins

Total Saponins was measured adopting modified Vanillin-sulphuric acid method by (Anh *et al.*, 2018). A standard curve was constructed using aescin, a natural triterpenoid saponin. Aescin, 150 mg was vortexed in 10 ml methanol to obtain an aescin stock solution. Methanol was utilised as the solvent blank and for preparation of the serial dilution of the standards in triplicate. 2 ml sample extracts, standard or methanol blank were taken and are placed in a water bath at 65°C for approximately 5 minutes to evaporate the methanol. 0.5 ml of vanillin, 2.5 ml of 72% v/v H₂SO₄ is added, mixed thoroughly and kept in a water bath at 60°C for 15 minutes for incubation. The absorbance was measured at 560 nm after cooling to room temperature

using imark Microplate Reader. The total saponins content was determined utilising Aescin standard curve and represented as mg of Aescin equivalents (AE) per gram.

Determination of tannins content

The Tannin content was measured utilising the protocol described by Makkar and Goodchild (1995). 200 mg of test sample is soaked in a conical flask containing 10 ml of aqueous acetone solution for fifteen hours. The tannin was collected as a supernatant in a flask using a Whatman filter paper No. 1. Fifty microlitre of supernatant (sample), standard was taken and made up to one ml using milliQ water and added 0.5ml of Folin Ciocalteu reagent, 2.5 ml of twenty percent Na₂CO₃, spin and incubated for 40 minutes at room temperature. Absorbance was measured at 725 nm using imark Microplate Reader. The Tannin content was determined utilising the Tannic acid standard curve and represented as milligrams of tannic acid equivalents per gram of sample (mg TAE/g) (Nassarawa *et al.*, 2019).

Evaluation of antioxidant activity

"1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging Assay" was performed using Dr Prieto's DPPH Microplate Protocol with minor modifications. 7.88 mg of DPPH powder is dissolved in 100 ml of methanol to obtain 0.2 mM DPPH Solution. Standard, test and control solutions are prepared. Ascorbic acid dissolved in methanol to give concentrations ranging from 0.2 mg/ml to 1.6 mg/ml are standard solutions (Xiang *et al.*, 2019). The extracts were dissolved in double distilled water to give concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mg/ml. Methanol will act as control. 100 µl of standard/test/ control solutions are taken in a 96 well microplate and added 100 µl of 0.2 mM DPPH. The plate is capped to reduce the evaporation and then covered in foil, kept at room temperature to shield the DPPH radical from deterioration by light, for thirty minutes. The absorbance (515 nm) is then read in imark Microplate reader (Rebai *et al.*, 2023). For each Extract Assay was run in Triplicate (Nguyen *et al.*, 2020). The Antioxidant Activity is determined utilizing the equation

% Antioxidant activity =

$$\frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Analytical statistics

Each experiment was performed in triplets. The data were analysed statistically using the statistical software (SPSS 12.0) and were presented as a mean ± standard deviation (SD). For comparison of concentration dependency, regression analysis was employed and one-way analysis of variance (ANOVA) was utilised for comparison of more than two means (Bhattacharjee *et al.*, 2023). When p value is less or equal to 0.05, a difference was judged statistically significant (Abdulhafiz *et al.*, 2022).

Phytochemical screening and analysis, determination of antioxidant activity were performed at DST-CURIE, Sri

Padmavati Mahila Visvavidyalayam, Tirupati andhra Pradesh from January 2021 to March 2022.

RESULTS AND DISCUSSION

Phytochemical screening

The results of phytochemical screening reveal the inherence of saponins, carbohydrates, phenols, glycosides, tannins, steroids, phytosterols, triterpenoids and diterpenes in all the aqueous and kamadhenu ark extracts of raw and sprouted little, kodo and proso millets. (Table 1 and 2). Alkaloids are present in KPM, KSKM, KLM, ALM and ASLM. Oils and fats are present in KPM, KSPM, KKM, KSKM, KLM and ASPM. The presence of phenols in aqueous and kamadhenu ark extracts of millets is in accordance to Liang *et al.* (2019).

Determination of total phenolic content

Phenols are potent antioxidants and also possess glucose lowering effect (antidiabetic). Total phenolic content TPC is shown in Table 3. KPM and KSPM has highest TPC of 48.34±1.5 and 32.68±2.2 mg GAE/g respectively. ALM and ASLM has the lowest TPC of 4.62±1.4 and 4.39±2.1 mg GAE/g respectively. Aqueous and kamadhenu ark extracts of proso and kodo millets possess high TPC in comparison to finger millet (2.61±0.02), pearl millet (4.79±0.01) and fonio millet (1.96±0.01) (Nassarawa *et al.*, 2019). Proso millets have more TPC than barnyard millet (16.07) (Ofosu *et al.*, 2020 and Kom *et al.*, 2020). Phenols are not present in foxtail millet (Sangma *et al.*, 2019). TPC of proso millet is in accordance to (Kom *et al.*, 2020). Polyphenols have been shown to reduce hyperglycaemia and enhance acute insulin production and insulin sensitivity in a number of different animal models and a few numbers of human trials. These characteristics signify the antidiabetic nature of millets. Rice samples had lower bound phenolic concentration than millet samples, indicating that millets are a best alternative of nutraceuticals. Antioxidant, anti-diabetic, microbicidal, bactericidal and antitumor properties are reported in bound phenolic compounds.

Determination of total terpenoid content

Terpenoids are plants synthesized small molecular products and are widespread group of natural products Murugan *et al.* 2022. Total terpenoid content TTC is tabulated in Table 3. KPM and KSPM exhibited highest percent of TTC 38.94±3.1 and 36.66±2.9 respectively. ALM and ASLM exhibited least per cent of TTC 3.94±2.2 and 4.68±2.4 respectively. Terpenoids are present in Foxtail millet Sangma *et al.* 2019. TTC of Finger millet, Pearl millet and Fonio millet are 11.92±0.01, 8.49±0.06 and 5.71±0.10 mg/g (Nassarawa *et al.*, 2019). Provides evidence for the presence of terpenoids in millets.

Determination of total saponin content

Saponins lowers the blood glucose levels by activation of glycogen synthesis and modulation of Insulin signalling.

Hence millets are consumed by people with diabetes and cardiovascular disorders. Total Saponin Content TSC is shown in Table 3. KPM and KSPM has highest TSC of 9.87 ± 2.2 and 9.78 ± 3.2 mg AE/g respectively. ALM and AKM has the lowest TSC of 2.98 ± 1.2 and 3.41 ± 1.5 mg AE/g respectively. Saponins are not present in foxtail millet Sangma *et al.* 2019. TSC of finger, pearl and fonio millets are 28.00 ± 0.04 , 38.64 ± 0.28 and 23.82 ± 0.18 mg/g

(Nassarawa *et al.*, 2019), thus possess rich TSC in comparison to proso, kodo and little millet.

Determination of tannins content

Medicinal Plants having Tannins Are Astringent, hence used for the treatment of Intestinal Disorders. Tannins Content TC is tabulated in Table 3. KPM and KSPM exhibited highest TC of 8.92 ± 1.6 and 8.66 ± 1.8 mg TAE/g respectively. ALM

Table 1: Phytochemical screening of kamadhenu ark extracts of millets.

Phytochemicals	KA raw	KA sprouted	KA raw	KA sprouted	KA raw	KA sprouted
	proso millet	proso millet	kodo millet	kodo millet	little millet	little millet
	KPM	KSPM	KKM	KSKM	KLM	KSLM
Carbohydrates	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Amino acids	-	-	-	-	-	-
Proteins	-	-	-	-	-	-
Oils and fats	+	+	+	+	+	-
Phenols	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Flavonoids	-	-	-	-	-	-
Alkaloids	+	-	-	+	+	-
Saponins	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Phytosterols	+	+	+	+	+	+
Triterpenoids	+	+	+	+	+	+
Diterpenes	+	+	+	+	+	+
Coumarins	-	-	-	-	-	-

Table 2: Phytochemical screening of aqueous extracts of millets.

Phytochemicals	Aqueous raw	Aqueous sprouted	Aqueous raw	Aqueous sprouted	Aqueous raw	Aqueous sprouted
	proso millet	proso millet	kodo Millet	kodo millet	little millet	little millet
	APM	ASPM	AKM	ASKM	AIM	ASLM
Carbohydrates	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Amino acids	-	-	-	-	-	-
Proteins	-	-	-	-	-	-
Oils and fats	-	+	-	-	-	-
Phenols	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Flavonoids	-	-	-	-	-	-
Alkaloids	-	-	-	-	+	+
Saponins	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Phytosterols	+	+	+	+	+	+
Triterpenoids	+	+	+	+	+	+
Diterpenes	+	+	+	+	+	+
Coumarins	-	-	-	-	-	-
Quinones	-	-	-	-	-	-

Note: + indicates the presence and - indicates the absence of phytochemicals.

All screening tests were carried out in triplicates.

and ASLM exhibited least TC of 1.98 ± 0.5 and 2.12 ± 1.1 mg TAE/g respectively. Proso millets are rich in tannins in comparative to finger millet, pearl millet and fonio millet with 7.70 ± 0.02 , 6.56 ± 0.02 and 5.92 ± 0.01 mg TAE/g (Nassarawa *et al.* 2019) barnyard millet with 1.05 mg TAE/g (Kom *et al.*, 2020).

Table 3: Total phenolic content (TPC), total terpenoid content (TTC), total saponin content (TSC) and tannins content (TC) in kamadhenu ark and aqueous extracts of millets.

Extract	TPC mg GAE/g	TTC Percentage	TSC mg AE/g	TC mg TAE/g
KPM	48.34 ± 1.5	38.94 ± 3.1	9.87 ± 2.2	8.92 ± 1.6
KKM	12.42 ± 1.8	11.62 ± 3.4	6.62 ± 1.9	6.48 ± 1.4
KLM	11.36 ± 2.5	11.45 ± 2.4	5.48 ± 3.4	5.32 ± 1.5
KSPM	32.68 ± 2.2	36.66 ± 2.9	9.78 ± 3.2	8.66 ± 1.8
KSKM	10.14 ± 1.6	10.48 ± 2.5	6.11 ± 2.6	6.34 ± 1.2
KSLM	6.62 ± 1.2	10.22 ± 1.9	5.86 ± 2.8	5.5 ± 1.6
APM	21.63 ± 2.4	16.23 ± 1.8	4.68 ± 1.6	3.14 ± 0.9
AKM	7.21 ± 1.6	6.72 ± 1.4	3.41 ± 1.5	2.62 ± 0.8
ALM	4.62 ± 1.4	3.94 ± 2.2	2.98 ± 1.2	1.98 ± 0.5
ASPM	20.34 ± 1.1	18.14 ± 1.5	5.43 ± 2.1	3.96 ± 1.7
ASKM	7.16 ± 1.8	8.19 ± 1.2	4.34 ± 1.8	2.74 ± 1.6
ASLM	4.39 ± 2.1	4.68 ± 2.4	3.65 ± 1.6	2.12 ± 1.1

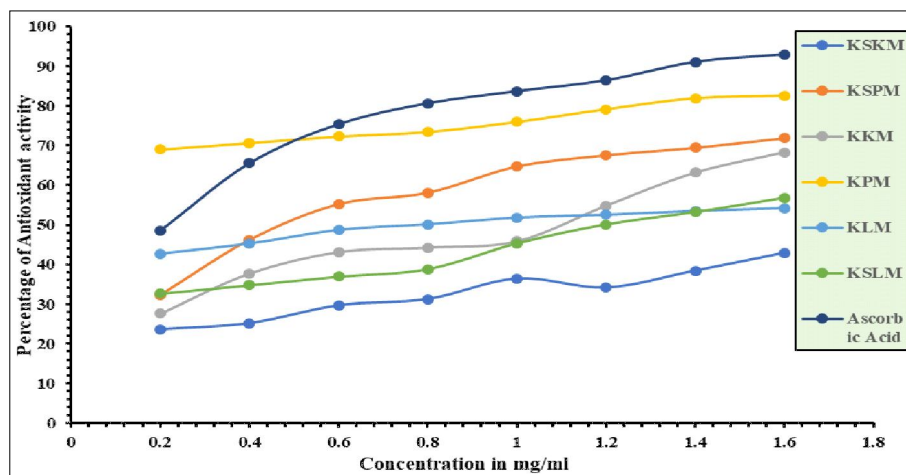


Fig 1: Comparative study of antioxidant potential of various kamadhenu ark extracts of millets with standard ascorbic acid.

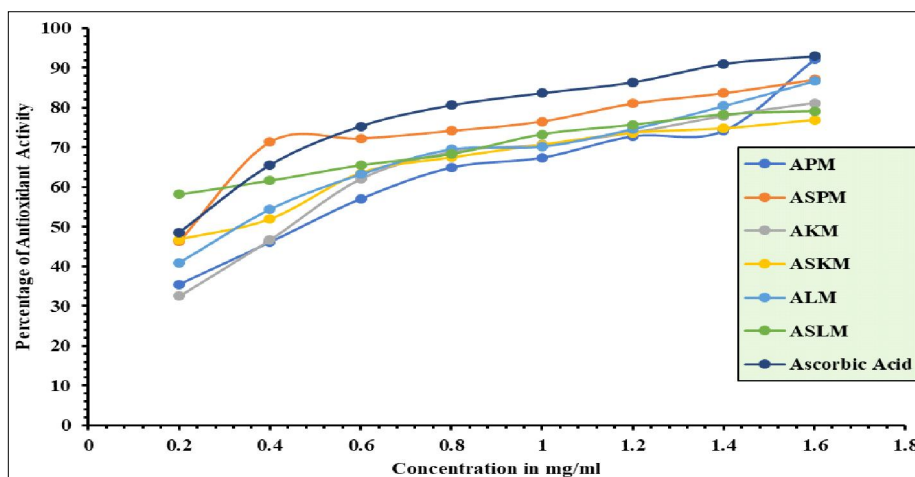


Fig 2: Comparative study of antioxidant potential of various aqueous extracts of millets with standard ascorbic acid.

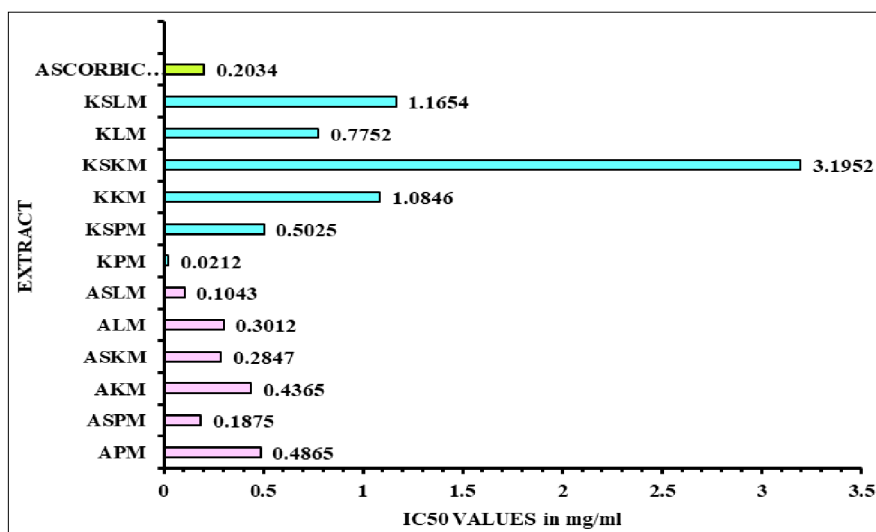


Fig 3: Comparative study of IC₅₀ values of various kamadhenu ark and aqueous extracts of millets with standard ascorbic acid.

Phytosterol esters significantly help to decrease the blood serum LDL cholesterol range by fourteen percent, however they have no impact on the blood serum HDL cholesterol range. The presence of Carbohydrates and Glycosides attribute for the delay in gastric emptying by Millets. Alkaloids have great potential to antimicrobial, anti-malarial and anti-inflammatory activities (Marella *et al.*, 2013). The steroids compounds are used to stress relive, decrease cholesterol level, induce immune system and increase memory power (Sharma *et al.*, 2011).

Determination of antioxidant potential

Present Study on determination of antioxidant potential adopting DPPH free radical scavenging activity reveals that KPM has the highest Antioxidant Activity of 69.03±0.12% and the least activity is shown by KSKM of 23.59±0.29% at 0.2 mg/ml. The IC₅₀ Values reveals that KPM has good antioxidant potential at very low concentrations of 0.0212 mg/ml and KSKM has least antioxidant activity with IC₅₀ Value of 3.1952 mg/ml (Fig 3). At lower concentrations the sprouted aqueous extracts have good antioxidant potential than raw aqueous extracts, the raw kamadhenu ark extracts have high Antioxidant potential comparative to sprouted kamadhenu ark extracts (Fig 1 and 2). Proso millet exhibits high antioxidant potential in comparative to finger millet, pearl millet and fonio millet with 67.06±0.04, 61.80±0.10 and 60.04±0.07 percent (Nassarawa *et al.*, 2019). Proso millet exhibits excellent antioxidant potential with IC₅₀ value of 0.0212 mg/ml in comparison to Barnyard millet, Italian millet and millet possessing IC₅₀ values of 0.3596, 0.4362 and 0.5543 mg/ml (Ofosu *et al.*, 2020).

CONCLUSION

In the present work antioxidant potential of aqueous and kamadhenu ark extracts of kodo millet, little millet and proso millet, are determined adopting DPPH free radical

scavenging assay. At lower concentrations the antioxidant potential is low and activity enhanced with increase in concentration. Kamadhenu ark extract of proso millet exhibited excellent antioxidant potential than kodo, little, barnyard, foxtail, finger, pearl and fonio millets. The Phytochemical analysis manifested the inheritance of carbohydrates, diterpenes, glycosides, phenols, phytosterols, saponins, steroids, tannins and triterpenoids in all the aqueous and kamadhenu ark extracts of raw and sprouted kodo millet, little millet and proso millet. Kamadhenu ark extract of proso millet is rich in total phenol content, total terpenoid content and tannins content than kodo, little, barnyard, foxtail, finger, pearl and fonio millets. Present *in vitro* studies should be confirmed *in vivo* and the phytochemicals should be isolated and used for further study.

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Conflict of interest

The authors have no conflicts of interest regarding this investigation.

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