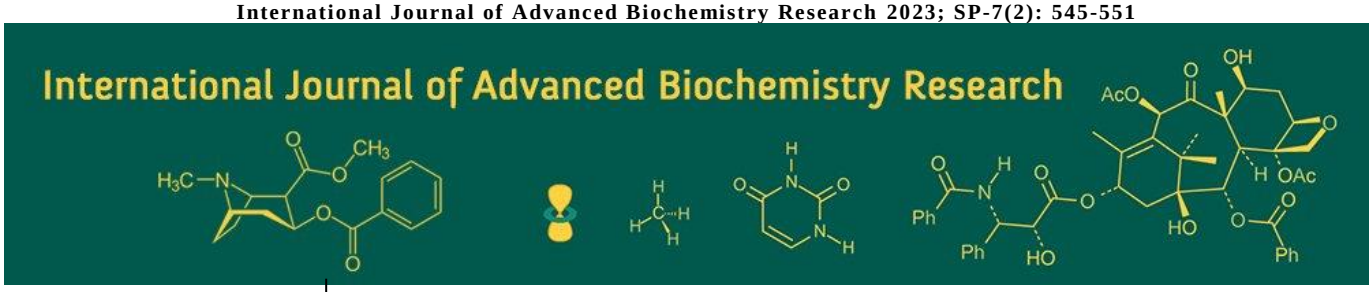

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Evaluation of millet proteins consumed in Indore region: Protein analysis and its digestibility assays

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Abstract
Millets have been a fundamental part of the Indian diet for centuries. They are extremely nutritious grains with substantial amounts of protein, carbohydrates, dietary fiber, antioxidants, minerals, and vitamins. This nutritionally packed grain once disappeared from the Indian staple diet is back as a superfood today due to its incredible health benefits. Millets contain more high-quality protein than major cereals such as rice and wheat. This makes millet a valuable food for people following vegan gluten-free diets. In this present analysis, two millets *viz*., Jowar (sorghum) and Ragi (finger millet) were evaluated for comparative protein analysis and their digestibility because of their larger consumption, production in India, and popularity among health-conscious people. Aqueous extracts of millet samples were prepared by homogenization following protein estimation and profiling by SDS-PAGE. The protein digestibility of millets was evaluated by Biuret method and agar well diffusion assay. The results confirmed that the protein content (14.5 mg/100 ml) and digestibility of Jowar were highest as compared to Ragi. Jowar protein digestibility by trypsin was prominent in the form of a clear zone diameter on the gelatin agar plate. SDS-PAGE analysis confirmed that Jowar showed maximum of 3 different types of protein bands ranging from 15 to 37 kDa. The consumption of cereal grains with high protein digestibility is of paramount importance to support growth and a healthy metabolism. It can thus be concluded that Jowar is an appropriate food grain to be incorporated into our daily diet due to its high protein quality and digestibility.

Keywords: Millets, protein, digestibility, SDS-PAGE, trypsin

Introduction
Millets are one of the ancient foods known to humans. India had a rich association with millets being the largest producer in the world. Millets have been a staple diet in rural India for years and remain prevalent even today [1]. They are India's very own superfoods and are considered extremely nutritious with health-promoting benefits [2, 3]. These are easier to digest and also superior to wheat and rice as they are rich in carbohydrates, protein, dietary fiber, good quality fat and have substantially higher amounts of minerals like calcium, potassium, magnesium, iron, zinc, and B complex vitamins [4, 5]. They have nutraceutical properties due to an abundance of antioxidants like polyphenols and flavonoids [6, 7].
Millets are coarse grains belonging to the botanical family Poaceae and come in two genres: major millets (jowar, bajra, ragi) and minor millets (KODO, foxtail, little millets, barnyard [8]. Jowar, bajra, and ragi are tagged as poor man's food due to their sheer affordability but the world is now noticing them for their enormous health potential [9]. These power-packed grains are known to protect cardiovascular health, control high blood pressure, cholesterol levels, and diabetes, and maintain a healthy weight with good digestive health [10]. India is the world leader in millet production with a 40% share with a current market of $9 billion [11]. It is expected to touch $25 billion by 2025. India produces 15.53 million tonnes of millet annually, contributing 10% to the country's food basket. The Government of India declared 2023, the International Year of Millets, to create awareness among people about its health and nutritional benefits globally [12].
Jowar is one of the ancient food grains and is a staple food crop in India and Africa. It has an abundant quantity of proteins, fiber, vitamins, calcium, and iron. Its dietary fiber decreases the chances of high blood pressure, cardiac diseases, and obesity and improves digestion. Its high magnesium content promotes cell regeneration and also increases calcium absorption thus it maintains blood calcium levels.

Its low glycemic index prevents a spike in blood sugar levels. Being a gluten-free grain, it is considered a safe food for people with celiac disease [13, 14].

Finger millet or Ragi is native to Africa and has a good amount of protein. Its amino acid profile differs from other millets as it contains Eleusinian amino acid which is biologically very important. Ragi is considered a notable reservoir of tryptophan, cystine, methionine, and other aromatic amino acids which are important for normal growth and development of the human body. Minerals like calcium, phosphorus, potassium, and iron are also present in Ragi which helps in maintaining bone density and increase level of hemoglobin. Ragi lowers blood pressure and bad cholesterol thereby reducing the risk of cardiovascular disease. It also controls sugar levels and thus diabetes by slow release of glucose in blood. Ragi is considered an ideal food for infants [15, 16].

The direct consumption of millets like Jowar and Ragi has significantly declined over the past decades due to a lack of awareness of their nutritional merits. A balanced diet that is high in protein is popular nowadays among vegans due to many health challenges. Millets Jowar and Ragi can provide healthy options for such people as these are light on the stomach, gluten-free, and rich in proteins. In this context, it is important to evaluate these two highly consumed millets for their protein content, quality, and digestibility to ensure their incorporation into daily diet. The study aimed to determine the total protein content, protein profiling, and invitro protein digestibility of Jowar and Ragi for comparative evaluation and future implications.

Materials and Methods

Sample collection

The two millets *viz*, Jowar and Ragi were purchased from the grain market in Indore. These samples (Fig.1) were cleaned and grains of uniform physiological shape and size were selected, for the present work.

A B

Fig 1: Seeds of A) Jowar and B) Ragi

Seed millets were weighed 2 gm and ground in a pestle mortar with 10 ml of 0.1 M potassium phosphate buffer (pH 7.4) to a fine slurry separately. The content was subjected to centrifugation at 5000 rpm for 10 minutes. After this supernatant was collected for further tests and labelled as millet protein extract.

Protein estimation

Total protein estimation was done using the Lowry method [17]. Aliquots of 25 microliters -400 microliter standard bovine serum albumin were taken in a series of test tubes using a micropipette. The volume taken was made up to 1 ml by adding the respective quantity of distilled water in each test tube. 200 microliters of each millet protein extract were taken in two separate test tubes. The alkaline copper reagent was added in a fixed volume (2.5 ml) to each test tube including blank. All the test tubes were allowed to stand at room temperature for 10 min. Colorimetric analysis was done by taking absorbance at 660 nm after 30 min against blank. A standard calibration graph was plotted between the absorbance and concentration of standard protein (BSA). The concentration of protein present in the sample was calculated from this graph. All the measurements were done in triplicates.

Protein profiling by SDS-PAGE

The millet protein extract was analyzed under reduced conditions using discontinuous SDS-PAGE [18]. The electrophoresis was conducted in 12% separating gel and 4% stacking gel. Approximately 10 ug of each millet protein was loaded separately in wells with 1:1 dilution with sample buffer (50 mM Tris pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.04% bromophenol blue) after boiling. Separation of protein bands was conducted at a constant voltage of 100 V. Following electrophoresis, the gel was stained with 0.2% (w/v) Coomassie brilliant blue R-250 made in 50% methanol/10% acetic acid. Gel was destained using 30% methanol and 10% acetic acid solution. The molecular weight of pulse protein was determined by using a pertained Protein Ladder having a mixture of 10 red, green, and blue dyed proteins (11-135 kDa).

Protein digestibility assays

All two millet protein extracts were tested for digestibility by the catalytic action of trypsin using Biuret method and agar well diffusion assay.

Biuret test

For Biuret method, an equal volume of millet protein test samples (200 ul) was treated with 200ul of trypsin (2 mg/ml) and incubated at 37 °C for 30 minutes [19]. After incubation addition of 5-7 drops of 0.5% $CuSO_4$ and 1 ml of 10% NaOH (Biuret reagent) was done for color detection. The appearance of light purple-violet color indicates a good level of protein digestibility by trypsin as compared to the control. Millet protein extract was taken as a control.

Agar well diffusion

Agar well diffusion assay was performed with slight modifications as described by Indumathy *et al.*, 2017 [20]. Only the digestibility of Jowar protein was taken under consideration for this assay as it showed the highest

digestibility upon application of the Biuret test. 200 ul of Jowar protein extract as substrate was added to cooled agar solution (1.5%) and poured into a petriplate. Wells were punched in this solidified agar plate supplemented with Jowar (protein. The different volume of trypsin enzyme (2 mg/ml) *viz*., 25 ul, 50 ul, 75 ul, and 100 ul was added into punched wells. For control, a phosphate buffer of pH 7.4 (50ul) was used. After an incubation period of 12 hours at room temperature plate was flooded with 10% TCA indicator solution for 5 minutes for better visibility. A clear zone around the well indicates a good degree of Jowar protein digestibility by trypsin. The overall study on Jowar and Ragi proteins is presented below in graphical form (Fig.2).

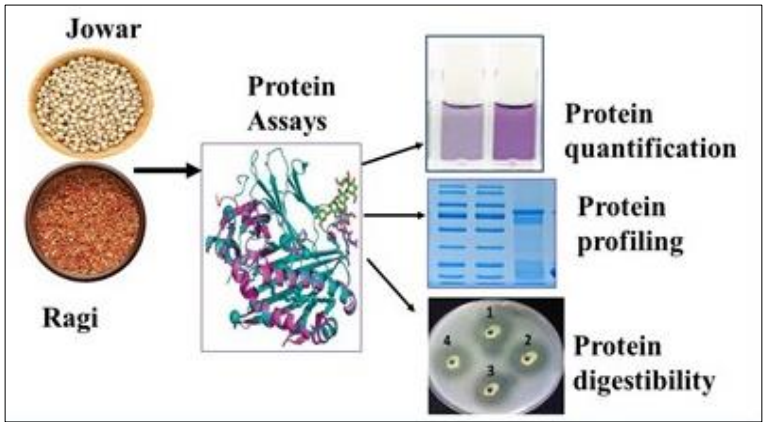


Fig 2: Overall experimental study on Jowar and Ragi proteins

Statistical analysis

All the experiments were carried out in triplicate and the results were given as the mean ± standard deviation (SD).

Results and Discussions

Yield of millet extracts

In this study, Jowar and Ragi were ground and then extracted for protein.

The following yields and color were obtained after the centrifugation of processed extracts of Jowar and Ragi as shown in Table 1 and Figure 3. Our results showed that pH 7.4 was able to extract a good amount of protein from millet grains. It has been reported that alkaline conditions can effectively extract proteins from cereals by making the protein more soluble [21]. The yield of Jowar extract was slightly higher than Ragi.

Table 1: Extraction yield of millet extracts

Sample	Final volume (ml)	Yield	Color of extract
Jowar	6.5	60%	Creamy white
Ragi	6.0	65%	Brown

Fig 3: Extracts of Jowar and Ragi

Protein content in pulses

Our findings (Table 2) confirmed that the quantity of protein in Jowar extract was found to be highest (14.5 mg/100 ml) while it was lowest in Ragi (10.5 mg/100 ml). The standard graph of BSA for protein estimation is presented in Figure 4. Our results are consistent with previous studies that reported the presence of 7-15% protein in Jowar and 5-8% protein in Ragi [22, 10]. The protein content of millet grains depends upon species, cultivar, growing conditions, and agricultural practices [23].

Table 2: Protein content in studied samples of millets

Sample	OD	Protein Content
Jowar	0.26	14.5 mg/100 ml
Ragi	0.11	10.5 mg/100 ml

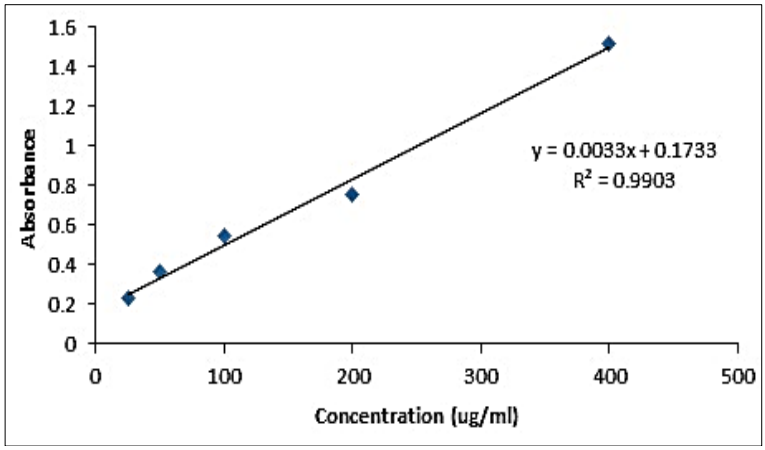


Fig 4: Standard graph of bovine serum albumin

Protein profiling of millets

The studies on the banding pattern on SDS-PAGE provide insight to identify and locate the presence of similar or variable proteins in the studied samples. A typical electrophoretogram under non-reduced conditions of marker and protein from Ragi and Jowar is given in Fig.4. The banding pattern illustrates mainly the presence of prolamin protein with molecular weight ranging from 10-37 kDa in both Jowar and Ragi samples. The greater number of bands ranging from 15-37 kDa with higher intensity in Jowar is indicative of its richness in protein quality and is thus considered to be a nutritious source of protein (Fig.5). Storage proteins are important components in the millets [24]. Proteins in millets are classified as albumins, globulins, prolamins, and glutelins [25]. Prolamins are major storage proteins abundant in all millets accounting for more than 50% of total seed protein [26]. The knowledge of structural properties of millet proteins is limited and further investigation can elucidate its interaction with other biomolecules in food. This can enhance their consumption and utilization for the manufacture of value-added food products. The total number of bands with relative molecular weight for the two millet samples is shown in Table 3.

Table 3: Number and molecular weight for observed protein bands in collected samples of millets

Sample	Number of bands	Molecular weight
Standard Protein Ladder	9	11-100 kDa
Jowar	3	15-37 kDa
Ragi	2	11-30 kDa

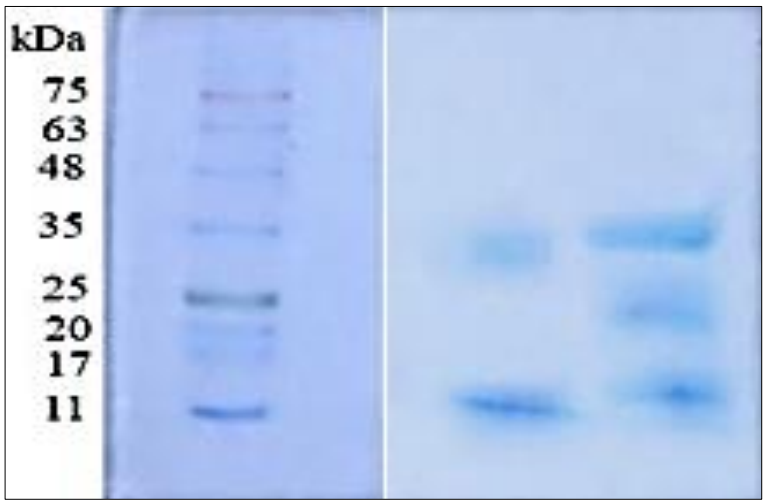
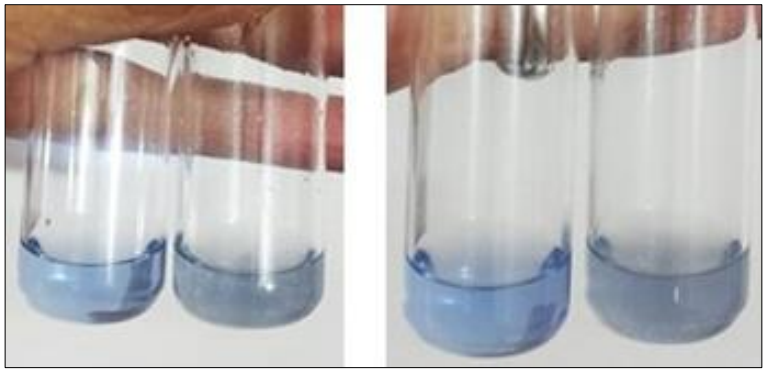


Fig 5: SDS-PAGE protein profile with protein ladder in collected samples of millets

Results for protein digestibility assays

Biuret assay for protein digestibility of two different varieties of millets after being incubated with trypsin revealed that Jowar showed maximum digestibility. The Jowar protein extract showed higher protein digestibility as compared to Ragi due to its light purple appearance (Fig.6A) as compared to the control (Fig. 6B) which demonstrates trypsin action on Jowar and Ragi protein.



Jowar Control
A

Ragi Control
B

Fig 6: Trypsin action on A) Jowar and B) Ragi

Among the two millet samples tested for protein digestibility by Biuret assay, only Jowar was selected for trypsin action on water agar medium due to its maximum digestibility. The result showed a zone of clearance (Fig.7) for a higher volume of trypsin with a zone diameter ranging from 1.0 to 1.2 cm (Table 4). This showed that Jowar protein extract had greater digestibility due to higher protein content than Ragi. The highest protein digestibility of Jowar by trypsin suggests its increased bioavailability since digestibility is an indicator of protein availability [27]. Highly digestible proteins have more nutritional value as they would provide more amino acids for absorption and protein synthesis. Several external and internal factors are responsible for protein digestibility like physical inaccessibility, amino acid sequence, protein folding, and crosslinking [28]. Since Jowar is gluten-free, therefore, it demonstrated significant protein digestibility. Gluten proteins contain high proline levels accounting for their limited digestibility [29].

Table 4: Zone diameter for Jowar protein digestibility by trypsin

S. No.	Volume of trypsin	Zone Diameter
1	25ul	Nil
2	50ul	Nil
3	75ul	1.0cm
4	100ul	1.2 cm

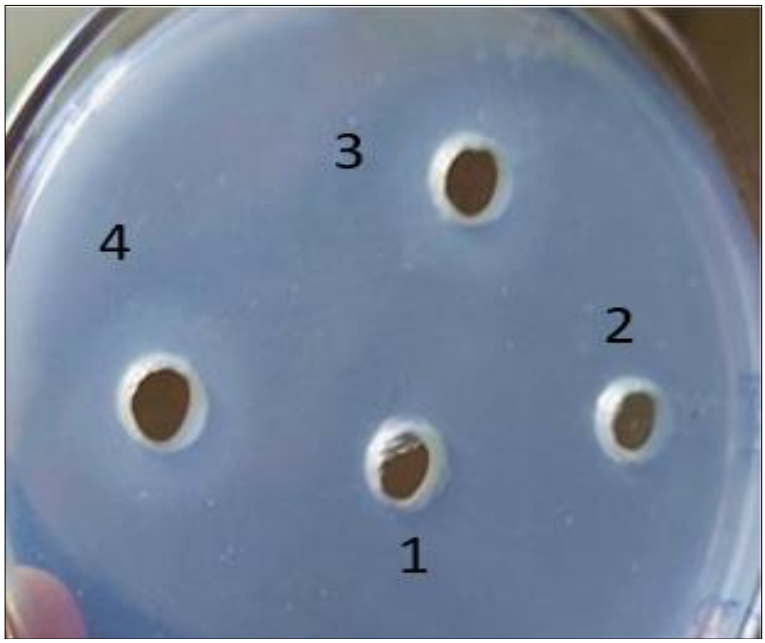


Fig 7: Protein digestibility of Jowar protein by well diffusion assay

Conclusion

Millets are a powerhouse of nutrients and contain protein as the second major nutrient. These are integral parts of the Indian diet not just because they have a good amount of protein, minerals, vitamins, and dietary fibres but also because of their affordability and availability. There is a growing demand for natural plant-based protein due to soaring prices of animal-based protein food. Millets can tackle the global challenges of sustainable food production, climatic changes, water scarcity, and alarming population in solving the problem of protein malnutrition. Moreover, millets are now gaining tremendous popularity, and health and wellness have become our supreme priority. The current study thus evaluates the most widely consumed millets like Jowar and Ragi for their total protein content, quality, and digestibility. The investigation reveals that Jowar represents a good source of protein with high content. The three protein bands in Jowar on electrophoretogram confirmed that it has a good concentration of different types of proteins. It also showed excellent protein digestibility by

trypsin than Ragi. Based on all these observations it is clear that Jowar is highly nutritive due to the presence of a significant amount of easily digestible protein. Therefore, Jowar is highly valued and considered best for health and fitness enthusiasts.

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