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Impact of Germination Time on Protein Solubility, Digestibility and In-Vitro Antioxidant, Anti- inflammatory Activity of Sorghum Grains

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Abstract: The present work was designed to obtain information on the effect of germination time on Protein Solubility, Digestibility and In-Vitro Antioxidant, Anti- inflammatory Activity of sorghum grain to evaluate the advantage compare to raw sorghum grain. Sorghum grains were germinated 24h to 96h at room temperature, Protein solubility and digestibility determined using crude samples and samples extracted with methanol was used for in-vitro antioxidant and anti-inflammatory assays. Protein solubility and digestibility was maximum at 96h 4.9mg/100g and 69% respectively. Amount of phenols maximum at 48h (661mg GAE/100g) and flavonoid contents increased after 48h germination and maximum at 96h (132mg RE/100g). Antioxidant and Anti-inflammatory activity showed maximum activity at 48h for DPPH (58.2%), ABTS (66.8%), FRAP (0.64 at 700nm) and hemolysis inhibition (48.16%) compared to standards Butylated hydroxyanisole (76.4%), Troxol (82%), Tannic acid (0.959 Abs at 700nm) and Acetylsalicylic acid (96.06%) drug respectively. Antioxidant activity is comparable to phenol content, increased during germination and decreased after 48h noticed. With this study scenario study conclude that the protein solubility and digestibility increased as germination and antioxidant and anti-inflammatory activity due to polyphenol content is out come with 48h germination used for pharmaceutical related activity is justified.
Keywords: Germination, Digestibility, Antioxidant, Anti-inflammatory activity.

Introduction

Sorghum (*Sorghum bicolor* [L.] Moench) is recognized as an important crop throughout the arid tropical and sub-tropical regions of Africa, Asia, Central America and other semi-arid regions of the world [(1)]. Given its natural tolerance to heat and drought stress, sorghum is a key crop in providing food security for millions of people in these regions[(2)]. In many parts of the country the crop is wholly utilized[(3)] and one of the most important weaning foods in low-income and high-income countries[(4)]. It ranks fifth among the world cereals, following wheat, maize, rice and barley in production area and total production. The nutrient composition of sorghum indicates that it is a good source of energy, proteins, carbohydrates, vitamins and minerals [(5)]. Sorghum contains good quality proteins are those that are readily digestible and contain the essential amino acids in quantities that correspond to human requirements [(6)].

Sorghum grain is known as a good source of phenolic compounds with a variety of genetically dependent types and levels, including phenolic acids, flavonoids and proanthocyanidins (condensed tannins). Not all sorghums contain proanthocyanidins, but all contain phenolic acids and flavonoids. Pigmented sorghums contain anthocyanins, namely 3- deoxyanthocyanidins (apigininidins and luteolinidins) that could be potential food colorants. Some sorghums have a prominent pigmented testa that contains proanthocyanidins, which are polymers of flavan-3-ol units with variable degree of polymerization[(7)]. Nowadays, there is a prominent interest on food and drinks that contain antioxidant molecules such as phenolic compounds[(8)]. Indeed, phenolic compounds have been reported to inhibit the development of cancerous tumours, and to have anti-bacterial, anti-inflammatory, antispasmodic and anti-diarrhoeic properties[(9)]. Most bioactivities of phenolic compounds are believed to be highly linked to their antioxidant activities. Since the environment and food processing have an impact on the levels of phenolic content, it is important to assess these parameters in some final sorghum foods [(10)]. In addition to the high content of anti-inflammatory phenolic compounds, sorghum contains several groups of bioactive compounds with the capacity to induce pro-inflammatory immune responses. Water-soluble beta-glucans are found in sorghum[(11)] that showed biologically active betaglucans capable of initiating macrophage activation.

Sorghum components, especially its protein is less digestible than other cereals for human and monogastric animals, because of its anti-nutritional factors such as tannins and phytic acid. Removal of these undesirable components is essential to improve the nutritional quality of sorghum and effectively utilize its potential as human food or animal feed[(12, 13)]. The low digestibility of sorghum proteins is presumably due to the high protein cross linking. Good quality proteins are those that are readily digestible and contain the essential amino acids in quantities that correspond to human requirements [(14)].

Germination is widely used in legumes and cereals to increase their palatability and nutritional value, particularly through the breakdown of certain antinutrients, such as phytate and protease inhibitors [(15, 16)]. Germination is a common practice in sorghum producing areas. Grains are malted for the production of weaning foods, opaque beers and other traditional dishes. Germination triggers the enzymatic activity of sprouting grains, leading to the breakdown of proteins, carbohydrates and lipids into simpler forms. This processing method activates proteases which are active in degrading proteins, thereby increasing nutrient bioavailability [(17)].

The objective of this study was to enhance sorghum nutritional value via germination, identify of sorghum protein characteristics, such as protein solubility, digestibility and antioxidant and anti-inflammatory activity of methanolic extract.

Materials and Methods

Sample

Grain sample sorghum collected from local market in Bangalore through Pristine Laboratories during January 2013. Sample grains were keeping in polythin bag and stored at 4^0C.

Chemicals

Pepsin, Pancreatin, α-amylase was obtained from Sigma– Aldrich. Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Gallic acid, Butylated Hydroxytoluene (BHT), Rutine and FeCl3 were purchased from Sigma Chemical. Sodium Carbonate from Merck Chemical Supplies. All other chemicals used were of analytical reagent grade.

***Germination* of grain**

Sorghum grains were soaked in distilled water for 20h with a ratio 1:5 w/v and the soaked water changed twice. At the end of soaking period, the soaked water was discarded. Soaked grains were germinated in dark at room temperature. Every day up to five days grains were collected regularly and dried. The root and shoot portions were manually removed. The grains were milled into fine flour and kept in polythin bag until analysis and stored at 4^{o} C.

Determination of crude protein

Crude protein contents of raw sorghum and treatments were determined according to the methods of A.O.A.C. [18].

Determination of protein solubility

Protein solubility was determined by the method of Sathe and Salunkhe [19]. One gram of samples was dispersed in 25 ml of 1 M NaOH. The obtained suspensions were mixed and stirred in an orbital shaker at 150 rpm for 12 h at room temperature and then centrifuged at 3000 g for 20 min. Soluble proteins in supernatants were determined by Bradford method [20]. Bovine Serum Albumin was used as standard protein. Soluble protein was expressed as g/100 g DW sample.

Determination of in vitro protein digestibility

In vitro protein digestibility was determined according to the method of Akeson and Stahmanna [21]. One gram samples added to HCl (15 ml, 0.1 M), containing 1.5 mg pepsin then the incubated at 37uC for 3 h. The obtained suspension was neutralized with NaOH (7.5 ml, 0.2 M), then treated with 4 mg of pancreatin in 7.5 ml 0.2 M phosphate buffer (pH 8.0). One milliliter of toluene was added to prevent microbial growth and the mixture was gently shaken and incubated for additional 24 h at 37uC. After incubation, the sample was treated with 10 ml of 10% TCA to remove undigested protein and larger peptides and centrifuged at 50000 g for 20 min at room temperature. Protein in the supernatant was estimated using the Kjeldahl method (A.O.A.C.) [18]. The percentage of protein digestibility was calculated by the ratio of protein in supernatant to protein in sample as equation:

$$\text{Protein digestibility \%} = \frac{\text{Nitrogen in supernatant-Nitrogen in blank}}{\text{Nitrogen in sample}}$$

Sample extract preparation

Ten g of finely ground samples were extracted in 200 ml methanol on a shaker at room temperature for 24 h. Subsequently, the extracts were centrifuged at 5000 rpm for 15 min and the supernatants were filtered through a Whatman No. 2 filter paper. The combined filtrate was evaporated at 40°C. The dried extract was weighed and redissolved in methanol to a concentration of 2 mg/ml then stored at 20°C until analysis.

Determination of Total Phenolic Content

The concentrations of phenolics in extracts were estimated using a modified spectrophotometric Folin-Ciocalteu method [22]. 1 ml of honey extract was mixed with 1 ml of FC reagent. After 3 min, 1 ml of 10% Na_2CO_3 solution was added to the mixture and made to 10 ml with distilled water. The reaction was kept in the dark for 15 min, after which the absorbance was read at 725 nm using a UV/VIS spectrophotometer (UV-1800, Shimadzu, Japan).The concentration of phenolic compounds was measured in triplicate. The results were expressed as mg of gallic acid equivalents (GAEs) per 100g.

Determination of Total Flavonoid Content

The total flavonoid content in extract sample was measured using the colorimetric assay developed by Zhishen [23]. 1 ml of extract was mixed with 4 ml of distilled water. Then 0.3 ml of $NaNO_2$ 5% w/v was added. After five min 0.3 ml of $AlCl_3$ 10% w/v was added followed by 2 ml of NaOH (1M) leave for 6 min. The volume was making up to 10 ml with distilled water. The mixture was vigorously shaken to ensure adequate mixing and the absorbance was read at 510 nm. The results were expressed as mg Rutine equivalents per 100g.

Free radical scavenging activity

The scavenging activity of DPPH free radicals developed according to the method reported by Gyamfi [24]. 50 µl of the extract in methanol was mixed with 1 ml of 0.135 mM DPPH in methanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4).

Methanol (50 µl) was used as the experimental control. After 30 min of incubation at room temperature, the reduction in the number of DPPH free radicals was measured, read the absorbance at 517 nm BHA was used as standard. The percent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample}]}{[\text{Absorbance of control}]} \times 100$$

ABTS radical scavenging activity

The scavenging activity of grains on ABTS (2,2´-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid) radical cation was estimated according to the method of Re et al [25]. Briefly, ABTS radical cation was freshly prepared by mixing 14 mM ABTS with an equal volume of 4.95 mM potassium persulphate and kept for 24 h in dark at room temperature. This ABTS radical cation solution was used for the assay after dilution in phosphate buffer saline (PBS) appropriately. To 50 µl of sample or standard, 150 µl of ABTS radical solution was added. After one min incubation at room temperature, the absorbance was measured at 734 nm. Methanol was used as a blank solution and ABTS solution without the sample served as a control. Trolox was used as a reference synthetic antioxidant compound. Reduction of ABTS radical in percent (R %) was calculated the same as described in DPPH radical assay.

Ferric-reducing antioxidant power (FRAP)

The FRAP (Ferric-reducing antioxidant power) of grain extracts was determined according to the method of Oyaizu [26] with some modifications. One ml of each sample was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide (K3Fe (CN) 6). The mixture was incubated at 50°C for 20 min and then 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was centrifuged at 1000 rpm for 10 min. The upper layer solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. A higher absorbance indicates a higher reductive capability.

Effect on hemolysis

Human Erythrocyte suspension

The whole blood was collected from a healthy volunteer had not taken any NSAIDS for 2 weeks prior to the experiment and collected in heparinzed vacutainer. The blood was washed three times with 0.9% saline and centrifuged simultaneously for 10 minutes at 3000 rpm. The packed cells were washed with 0.9% saline and a 40% v/v suspension made using isotonic phosphate buffer which was composed of 154mM NaCl in 10mM Sodium Phosphate Buffer at pH 7.4 used as Stock erythrocyte or RBC suspension.

Hypotonic solution-induced hemolysis

The membrane stabilizing activity of the extract was assessed according to the method described by Shinde [26] with slight modifications. The test sample consisted of stock erythrocyte (RBC) suspension 0.50ml mixed with 5ml of hypotonic solution (50mM NaCl in 10mM Sodium Phosphate Buffered saline at pH 7.4) containing extract ranging from concentration 20-100 µg/ml. The control sample consisted of 0.50ml RBC suspension

mixed with hypotonic buffered solution alone. The standard drug acetylsalicylic was treated similar to test at 10µg/ml concentration. The experiment was carried out in triplicate. The mixtures were incubated at 10 minutes at room temperature, centrifuged for 10 minutes at 3000rpm and absorbance of the supernatant was measured spectrophotometrically at 540 nm. The percentage inhibition of hemolysis or membrane stabilization was calculated by following equation.

% Inhibition of hemolysis = 100 x [A 1 –A 2 /A 1]

Where:

A 1 = Absorbance of hypotonic buffered solution alone

A 2 = Absorbance of test /standard sample in hypotonic solution.

Statistical Analyses

Assays were performed in triplicate, and the results were expressed as mean values with standard deviations (SD).

Results

Effect of germination of sorghum on crude protein

Table 1 presents crude protein content in sorghum before and after germination. Protein content in raw sorghum variety obtained 10.10±0.04%. Protein was significantly higher in raw at 0h. The crude protein was significantly decreased after germination 24 to 96h compared with raw sorghum ranged from 9.81±0.02 to9.03±0.03% respectively and this may be due to the activity of proteolytic enzymes.

Effect of germination of sorghum on protein solubility

Table 2 exhibits the effect of germination of sorghum on protein solubilized under alkaline conditions extracted with NaOH as described in materials and methods. Data showed that solubility of raw sorghum protein is 3.72±0.01g/100 g. There was a significant increase in protein solubility after germination 24 to 96h ranged from 3.96±0.01 to 4.90±0.01g/100g.

Table 1. Crude protein content of sorghum after germination.

Treatments in(h)	Crude protein (%)
Raw (0)	10.10±0.04
24	9.81±0.02
48	9.64±0.05
72	9.22±0.01
96	9.03±0.03

Results were expressed as mean values with standard deviations

Table 2. Protein solubility of sorghum after germination (g/ 100 g DW).

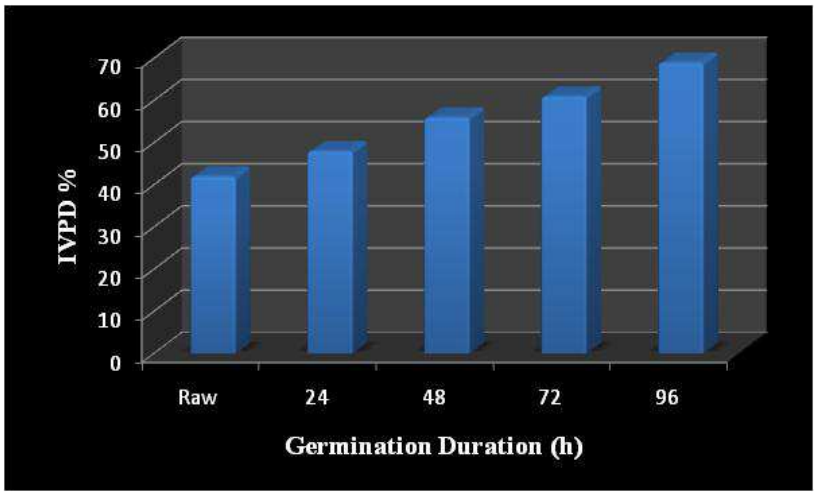
Treatments in(h)	Protein solubility
Raw (0)	3.72±0.01
24	3.96±0.01
48	4.18±0.01
72	4.53±0.02
96	4.90±0.01

Results were expressed as mean values with standard deviations

Effect of germination of sorghum on in vitro protein digestibility (IVPD).

Figure 1 presents the in vitro protein digestibility in sorghum before and after germination. Data showed that in vitro protein digestibility in raw sorghum was 42% and significantly increasing in protein digestibility followed the germination ranged from 48 to 69% at 24 to 96h respectively.

Figure 1. In vitro protein digestibility (IVPD%) of sorghum after germination.



Total phenolic content

Polyphenolic contents in methanolic extracts were expressed as mg gallic acid equivalents per 100 g of sample. There was a wide variation observed in the total polyphenol content among the sorghum treatment. The total polyphenol content of the sample raw was found to be 619.66± 0.68mg GAE/100g **(Figure 2).** There was a phenol variation in germinated samples. As germination goes on the total polyphenolic content increased up to 48h showing 661.06± 0.46mg GAE/100g maximum after that there is a decreased content of polyphenol was notified.

Total Flavonoid Content

The amounts of flavonoids recorded for all the samples investigated explains their biological functions against allergies, ulcers, inflammation, platelet aggregation and these biological functions. Raw sample having very less 96mg/100g. After germination there is a significant increase of flavonoid contents was showed (**Figure 3**). The content of flavonoid highest in 72h exhibited 141mg/100g. Results express as phenol content decreases the flavonoid content increase.The supportive information not available with respect to germination.

Figure 2. Total polyphenol contents in methanolic extracts of sorghum after germination.

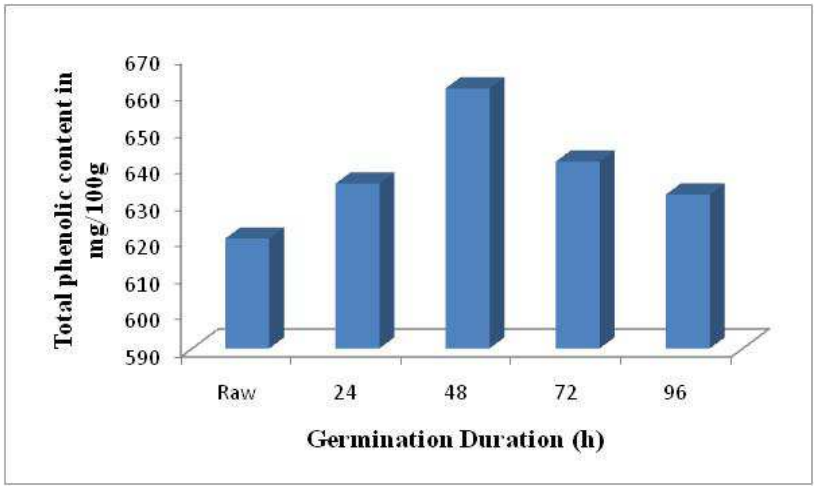
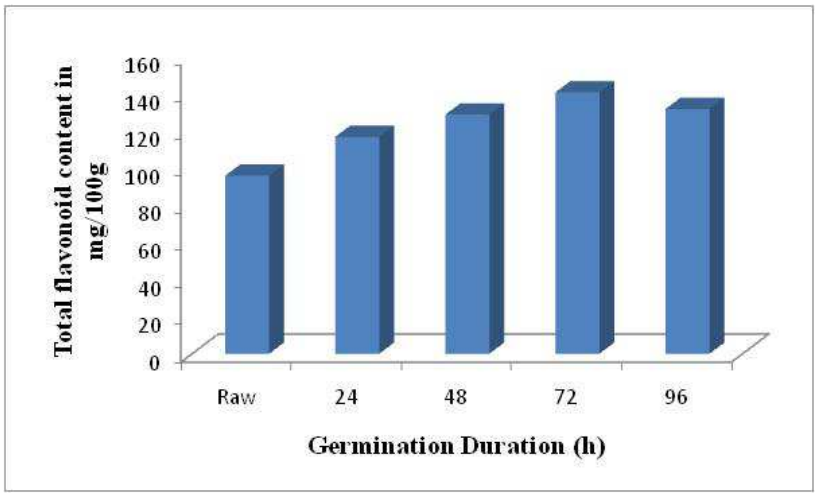


Figure 3. Total flavonoid content of methanolic extracts from germinated sorghum grains.



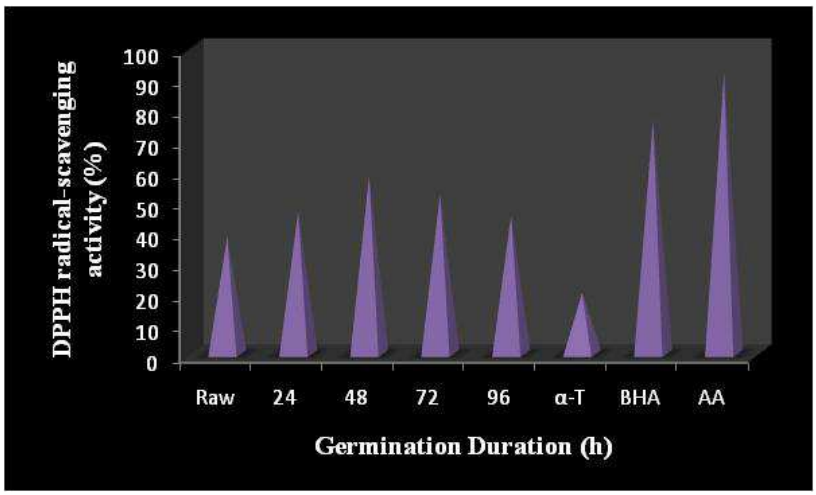
DPPH free radical scavenging activity

The DPPH free radicals scavenging activities of grain extracts, BHA, α-tocopherol and ascorbic acid are presented in **(Figure 4**). All of the extracts exhibited remarkable scavenging effect on the stable DPPH radical. Ungerminated raw grain showed 38% inhibition but all germinated grain extracts showed higher radicals scavenging activities ranging from 45.1 – 58.2% compare to α-tocopherol (19.8%), BHA (76.4%) and ascorbic acid (92.1%). Among the germinated grains at 48h found to showing higher activity, may be due to higher in total phenol content correlating the results obtained.

Scavenging of ABTS radical

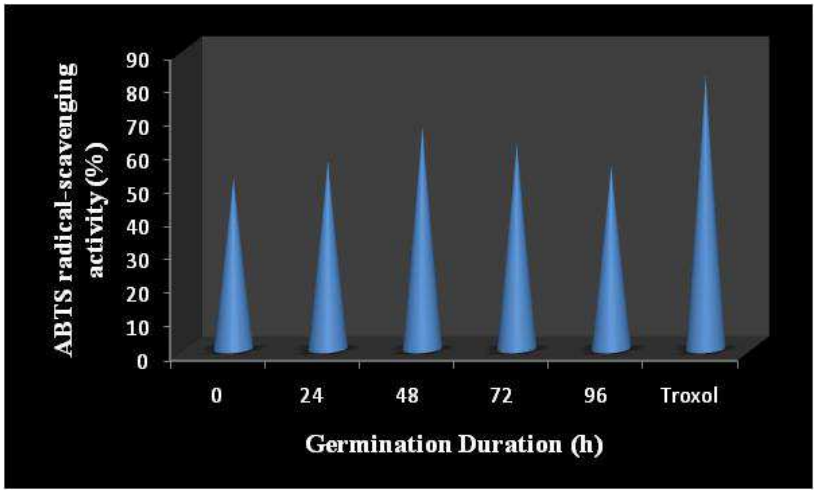
The capability of the grain extracts and trolox (antioxidant standard) to scavenge ABTS radicals is shown in **(Figure 5). The** abilities of the tested samples to scavenge ABTS radicals were compared to trolox standard. Raw crude extract exhibited good antioxidant activities (51.4%) compared to trolox (82%). The raw sample was compared to the germinated at different hours, showing highest scavenging ability (66.8%) was exhibited at 48h and it is again correlating phenol content in the sample responsible for this activity.

Figure 4. Percentage inhibition of DPPH free radical of sorghum extracts after germination.



α-T: α-Tocopherol, **BHA:** Butylated hydroxyanisole, **AA:** Ascorbic Acid

Figure 5. Scavenging effect of germinated grain extracts and Trolox (as standard) on ABTS radical cation.



Reducing power activity

The reducing power of methanolic extracts is presented in **(Figure 6).** All tested samples showed strong ferric ion-reducing activities. Raw sample (0.52 at 700nm) compared to 48h germinated sorghum grain found to contain high 0.62 at 700nm and standard tannic acid (0.95 at 700 nm).This indicated that polyphenolics in methanolic extracts of sorghum grains may play a role as electron and hydrogen donors.

In-vitro anti-inflammatory activity

This study reports the anti inflammatory activity of methanol extract of sorghum grain by *in vitro.* The raw extract was less effective in inhibiting the hemolysis showed only 30.11%. But effect of germination on hemolysis protection was studied and showed significant to germination rate. At 48h germination found to contain high 48.16% comparable to standard drug acetylsalicylic acid showed 96.06% of protection (**Figure 7).** These provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect.

Figure 6. Reducing power of methanolic extracts from germinated sorghum grains.

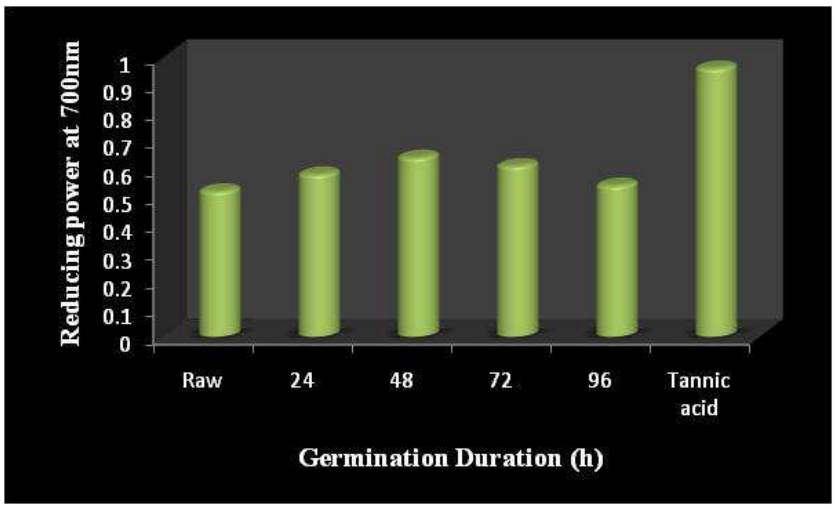
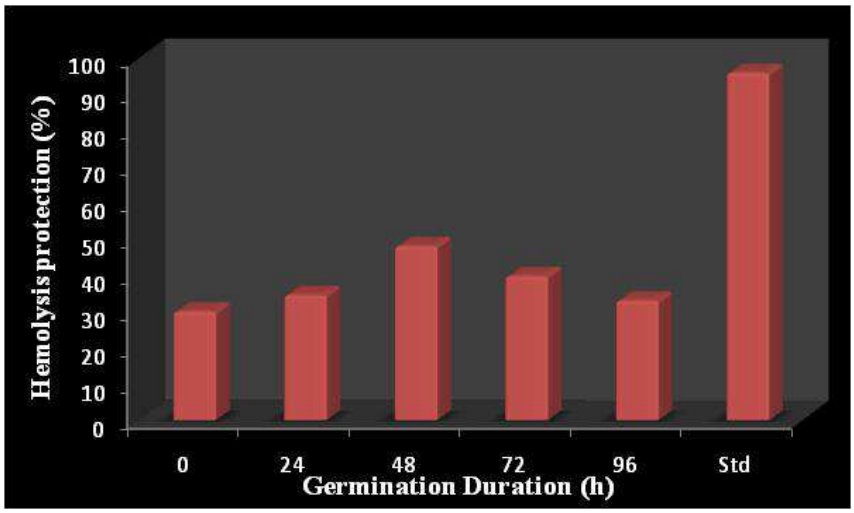


Figure 7. In-vitro anti-inflammatory effect of germinated sorghum extracts.



Discussion

The obtained crude protein in our results agreement with Dicko[28] and Johnson[29] who found that crude protein content in whole sorghum grain is ranged from 7 to 15% or 10.30 to 14.90%. These results are agreed with Shaker [30] who reported that nutrients loss might be attributed to the leaching of soluble nitrogen, mineral and other nutrients into desired solution. Among the functional properties of proteins, solubility is probably the most critical function. Protein solubility characteristics are influenced by factors such as origin, processing conditions, pH, ionic strength and the presence of other ingredients [31]. As our germination time proceeds the solubility of protein also increased compare to raw at 0h. These findings are in agreement with Elkhalifa and Bernhardt [31], who found that germinated sorghum had a higher protein solubility compared with the un-geminated one. The protein of the germinated sorghum was more soluble than the ungeminated sorghum. This might be due to the high proteolytic activity during germination, which will lead to an increase in the protein solubility resulting from hydrolysis of the storage proteins. The factors that may affect sorghum protein digestibility, divided in two categories: exogenous factors (i.e. interactions of proteins with non-protein components such as polyphenols, starch, non-starch polysaccharides, phytates and lipids), and endogenous factors (factors arising from the sorghum proteins themselves), concluding that the poor digestibility of sorghum proteins appear to be multi-factorial. The main proteins in sorghum were kafirins. These proteins are known to be peptidase resistant because of their S-S bonds. Digestibility may be used as an indicator of protein availability [32]. Our study IVPD results are similar to Correia [33], who found that germination causes activation

of intrinsic amylases, proteases, phytases and fiber-degrading enzymes, thereby increasing nutrient digestibility. The activity of intrinsic proteases in germinated grains leads to an increase in-vitro protein digestibility. Germination is effective in increasing protein digestibility and improving sensory properties Also, processing of sorghum (boiling, germination, fermentation and cooking) greatly improved its nutritive value [34, 35].

Antioxidants in grains are difficult to be extracted due to different solubility of active compounds [36]. Among cereals, sorghum has the highest content of phenolic compounds reaching up to 6% (w/w) in some varieties [9]. Hence, it is important to quantify polyphenolic contents in sorghum grains and to assess their contribution to antioxidant activities. Reports on the levels of phenolic compounds in sorghum varieties show a high intervarietal difference of contents among varieties screened[37, 38]. However, levels of total phenols of sorghum reported in the literature vary widely due to differences in extraction solvents, test methods and standards used. This makes it difficult to do direct comparisons [39]. Flavonoids are the largest group of polyphenolic compounds found in higher plants and synthesized from the shikimic acid and malonic acid pathways [40].

Flavonoids possess free radical scavenging activities which prevent oxidative cell damage, have anti-inflammatory, anticancer activities as well as protection against the different levels of carcinogenesis. In our present study phenol and flavonoid contents representing germination variation very well. The stable DPPH radical, which has a maximum absorption at 517 nm, is widely used to evaluate the free radical scavenging activity of hydrogen donating antioxidants in many plant extracts[41]. Dlamini [42]reported that tannin sorghums, Red Swazi, NS 5511 and Framida, had significantly higher ABTS and DPPH antioxidant activity when compared to sorghums without a pigmented testa layer, Macia and NK 283. The consumption of sorghum based foods can be beneficial in preventing oxidative stress related degenerative diseases. The ABTS method is widely employed for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidants in many plant extracts [43]. Hagerman [44] have reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals (ABTS•+) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific functional groups. Again contribution of germination effect can be well executed through studying DPPH and ABTS assays showing quenching activity of oxidative stress responsible radicals generated and nullified by our raw and germinated sorghum extracts.

Miller[36] reported that the antioxidant capacities of cereals and cereal products are high, especially those of wheat, corn and sorghum. On a fresh matter basis, are even equal or higher than those of some fruits and vegetables[44]. Inflammation is very common symptom of many chronic diseases. It is a normal protective response to tissue injury caused by physical trauma, noxious chemical and microbial agents. Inflammation is a protective response by the body to remove injurious stimuli as well as initiate the healing process for the tissue[45].Our results also representing membrane stabilizing property by inhibiting damage from hypotonic solution (Figure 7).

According to the results of this study, it can be concluded that nutrional value and functional properties can be improved by germination can be declared in this study. The methanolic extracts of sorghum have significant antioxidant activities against various antioxidant systems *in vitro* and accessible source of natural antioxidants can be used as a possible food supplement or in pharmaceutical industry. Polyphenol contents seem to be the main components responsible for the antioxidant activity and anti-inflammatory activity of all grain extracts is comparable. Results suggest that germination can key process for food quality and functional property improvement can be justified.

Conflict of interest statement

We declare that we have no conflict of interest.

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