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Analytical Methods

Using combined optimization, GC–MS and analytical technique to analyze the germination effect on phenolics, dietary fibers, minerals and GABA contents of Kodo millet (*Paspalum scrobiculatum*)

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ABSTRACT

A central composite rotatable design was applied to study the effects of soaking time, germination time and temperature on the responses; total phenolics, total flavonoids and antioxidant activity for the biochemical enhancement of bioactive components of Kodo millet. The optimum conditions for producing germinated Kodo millet flour of highest TPC (83.01 mg GAE/100 g), TFC (87.53 mg RUE/g) and AoxA (91.34%), were soaking time (13.81 h), germination temperature (38.75 °C) and germination time (35.82 h). Protein increased significantly form, 6.7 to 7.9%, dietary fibers from 35.30 to 38.34 g/100 g, minerals from 232.82 to 251.73 mg/100 g, GABA contents from 9.36 to 47.43 mg/100 g, whereas phytates and tannins decreased from 1.344 to 0.997 mol/kg and 1.603 to 0.234 mg/100 g respectively, in optimized germinated Kodo millet sample. Six new bioactive compounds [*n*-propyl-9,12,15-octadecatrieno ate (0.86%), pregan,20-one-2 hydroxy,5,6,epox-15-methyl (3.45%), *hexa*-decanoicacid (8.19%), 9,O-ctadecenoicacid (5.00%), butyl-6,9,12,15-octadecatetraenoate (4.03%), hexadecanoicacid-methylester (1.43%)], synthesized as a result of germination under optimum conditions in the Kodo millet depicted the germination potential of millets as a source of valuable bioactive compounds.

1. Introduction

Kodo millet (*Paspalum scrobiculatum*) is an important minor millet crop predominantly grown in the Indian subcontinent (Takeshima & Nagarjan, 2012). Previous studies have revealed that the Kodo millet grain extracts possess high *in vitro* antioxidant capacity, where as the whole grain of Kodo millet has shown a high anti-diabetic effect in alloxan-induced diabetic rats (Prashant, Hegde, & Chandra, 2005). Millets are significantly rich in resistant starch, soluble and insoluble dietary fibers, minerals, and antioxidants (phenolic acids, glycated flavonoids and nutraceuticals). Malleshi and Hadimani (1993) have reported that the Kodo as well as little millets (*Panicum miliare*) contain about 37–38% of dietary fiber content, which is the highest among the cereals, whereas, their fat contain higher amount (4.2%) of poly unsaturated fatty acids.

During the process of germination of cereals, a significant change in their biochemical, nutritional and sensory characteristics has been reported due to the degradation of reserve materials, since they are used for respiration and synthesis of new cell constituents of developing embryo in the seed (Danisova, Holotnakova, Hozova, & Buchtova, 1995). In recent years, some researchers have revealed that the consumption of sprouts is a new way of nutritional enhancement and has received large attention in the development of functional foods, because of increase in nutritive value due to increase in vitamins, fiber, trace elements, amino acids, flavonoids as well as phenolic acids after sprouting. Reports also indicated that, as compared to un-germinated seeds, germinated seeds contain more protein, low levels of unsaturated fatty acids and have less carbohydrate (Narsih, Yunianta, & Harijono, 2012; Perales-Sanchez et al., 2014). Grewal and Jood (2006) reported that the mineral contents such as phosphorus, calcium, zinc and copper increases in sprouts as a result of hydrolysis of phytic acid due to increase in the phytate enzyme activity as a result of germination.

Abbreviations: AoxA, antioxidant activity; CCRD, central composite rotatable design; GAE, gallic acid equivalents; GT, germination temperature; Gt, germination time; RSM, response surface methodology; RUE, rutin equivalent; ST, soaking time; TFC, total flavonoid content; TPC, total phenolic content; GABA, *gamma*-amino butyric acid.

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It is believed that sprouts are rich in health-promoting phytochemicals as compared to their un-sprouted counterpart (Hübner & Arendt, 2013). Some researchers have suggested that for promoting the nutraceuticals quality of cereals, pseudocereals and legumes, germination (sprouting) is the most inexpensive and effective process (Alvarez-Jubete, Wijngaard, Arendt, & Gallagher, 2010; Botero, Fong, Rothschild, & Patrick, 2012; Pasko, Sajewicz, Gorinstein, & Zachwieja, 2008; Perales-Sanchez et al., 2014). Antioxidants such as polyphenols and flavonoids are one of the most common active ingredients of nutritional and functional foods. They can contribute an important role in human health in the prevention of oxidation and cellular damage by inhibiting or delaying the oxidative processes. The antioxidant activity of these compounds increases as a result of germination bioprocess. This is one of the reasons, that the consumption of sprouts is considered very important in reducing human diseases associated with oxidative stress (Pasko et al., 2009; Silva, Pereira, & Azevedo, 2013).

GABA (γ-amino butyric acid) is currently an interesting compound which increases during germination via protein metabolism of seed components (Park et al., 1999). Shiahs and Yatham (1998) have reported that γ-amino butyric acid directly affects the personality and the capability of a person to manage stress and acts as a neurotransmitter in the brain. Similarly the importance of determining the free and bound phenolic compounds in the food materials has been highlighted by Adom and Liu (2002).

For optimizing the complex processes, RSM is an efficient statistical technique. The main advantage of RSM is that it reduces the number of experimental trials needed to evaluate the multiple parameters and their interactions with are considered laborious and time-consumption (Irakoze, Haihua, Qin, Zhou, & Huiming, 2010). Chandrika and Fereidoon (2005) reported that the extraction process variables, such as anthocyanins, phenolic compounds, polysaccharides, vitamin-E and protein from varied materials can be optimizing by using the RSM optimization technique.

Kodo millet is reported to have the highest nutritional attributes among the minor millets but due to the presence of high amount of phytates and other anti-nutritional factors, their bioavailability is hampered. Germination has variable responses on seed components concentration, bioavailability and the biosynthesis of new components, depending upon the raw material used. The authors have adopted a similar approach previously to analyze the effect of germination on various characteristics of barnyard millet constituents and promising results were obtained. Since a very few work has been carried out in this current and important area of research, the present work highlights the different biochemical modifications that possible can occur in Kodo millet grain during soaking and germination under optimum conditions which possibly can improve its nutritional quality, make it acceptable as functional food ingredient and as raw material for infant food development. A three way approach including optimization, analytical and GC–MS analysis were adopted to establish the optimum germination conditions in order to enhance the bioavailability of total phenolic, total flavonoid and GABA compounds so that their *in vitro* anti-oxidative capacity can be improved. Similarly the effects of soaking and germination on other functional ingredients such as protein, dietary fiber, minerals contents and antinutritional factors were also taken into consideration to explore the minor millet seeds application area.

2. Material and methods

2.1. Procurement of raw material

Kodo millet (*Paspalum scrobiculatum*) was purchased from authorized seed centre located in Hyderabad (India). All the reagents and solvents used in this research were of analytical and HPLC grade. Reagents such as sodium nitrite, aluminium trichloride, ascorbic acid, were purchased from Standard Instruments Corporation, Pvt. Ltd., Patiala (India). The solvents purchased from Merck Chemical Pvt. Ltd., Shiv Sagar Estate A. Worli Mumbai-400018, were having percentage purity of 99.8% and 99.5%. The chemicals and reagents such as Folin-Ciocalteu reagent, 2,2′-diphe nyl-1-picrylhydrazyl (DPPH), gallic acid, rutin, potassium ferricyanide, potassium phosphate dibasic and monobasic (used in making phosphate buffer) needed for determining antioxidant activity were purchased from Sigma-Aldrich, India.

2.2. Preparation of germinated Kodo millet flours

Kodo millet seeds were washed and soaked in distilled water at room temperature for variable time intervals (8–16 h). Soaked seeds were germinated in a pilot scale seed germinator (Macro Scientific Works, Pvt. Ltd., India) at different temperature (19.55–46.55 °C) and time period (13.20–46.80 h). A relative humidity of 80–90% within the chamber was maintained by using automatic overhead water supply reservoirs. The resultant bio-processed Kodo millet seeds were dried at 45 °C for about 8 h to a final moisture content of 7–8%, then cooled down to 25 °C. The raw and germinated millet seeds were ground in a lab grinder (Agrosa Pvt. Ltd, India). The resultant flour samples were passed through 100 mesh sieve to obtain uniform millet flour and finally packed in air tight containers and kept at 4 °C for further analysis.

2.3. Statistical analysis and optimization

The RSM was used to derive the optimum levels of the independent variables using a three factor, five level central composite rotatable design (CCRD) which dictated 20-experimental combinations including six replicates at the centre point (Table 1). The independent variables chosen were soaking time (ST), germination temperature (GT) and germination time (Gt), including three responses: total phenolic contents, total flavonoids contents and *invitro* antioxidant activity with respect to germinated Kodo millet flour. The data were analyzed by multiple regression equations using the least-squares method. A second order polynomial equation was used to express the responses as a function of the independent variables (n) as given below.

$$Y_k = \beta_{k0} + \sum_{i=1}^{n}\beta_{ki}x_i + \sum_{i=1}^{n}\beta_{kii}x_i^2 + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\beta_{kij}x_i x_j$$

where: Y_k = response variable; Y_1 = total phenolic content (mg GAE/100 g); Y_2 = antioxidant activity (%); and Y_3 = total flavonoid content (RUEmg/g). x_i represents the coded independent variables; x_1 = soaking time, $x_{2=}$ germination temperature, x_3 = germination time, whereas, β_{ko} indicates the value of the fitted response at the centre point (0, 0, 0) of the design. β_{ki}, β_{kii} and β_{kij} represent the linear, quadratic and cross-product regression coefficients, respectively. The test of statistical significance was performed on the total error criteria, with a confidence level of 95%. For each response the significant terms in the model were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test comparison among means with 5% of significance level. The statistical software Design Expert version 7.0.0 (Stat-Ease, Minneapolis, MN, USA) was used for RSM analyses.

2.4. Analysis of chemical constituents

The chemical constituents such as protein and lipid contents of the Kodo millet flours (raw & optimized germinated samples) were determined by AOAC (2012) methods. Minerals (calcium, magne-