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"Evaluation of Nutraceutical properties of *Amaranthus hypochondriacus* L. grains and formulation of value added cookies"

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Abstract
In the present study, the dried *Amaranthus hypochondriacus* (L.) grains were investigated for their phytochemical content, antioxidant and antimicrobial activity to understand the nutraceutical properties. A thorough nutritional characterization of the dried Amaranth grains demonstrated them to be a good source of natural antioxidants and minerals like Phosphorus, Calcium, Magnesium and Iron followed by the other nutrients. Phytochemical analysis of the dried grains revealed the presence of good phenolics, flavonoids, alkaloids and saponins content. No antimicrobial activity was observed in the methanolic extract against the three gram positive and one gram negative test bacterial pathogen. Fortified cookies were formulated using the Amaranth grains, oats and the refined wheat flour, for nutritional analysis. From the nutritional analysis, it was observed that the cookies can act as a good source of protein, carbohydrates, and dietary fiber and hence a potential source of energy.

Keywords: *Amaranthus hypochondriacus* L, Phytochemical, Antioxidant, Antimicrobial, Nutritional.

1. Introduction
The Amaranth, commonly known as rajgira (king seed), ramdana (seed sent by God) is known to be a very nutritious pseudocereal with an exceptionally high protein content as compared to the true cereals. Unlike most conventional grains such as wheat, rice and corn which are low in lysine, the Amaranth makes an attractive source of protein because, if consumed along with other cereals, it can provide a ''balanced'' protein content. So, the interest in the use of Amaranth flour in blends with wheat or maize has increased over the recent decades. The Amaranth species are also receiving a great attention in the developing countries as a means to overcome protein malnutrition. Tocopherols in the Amaranth seeds include c- and d-tocotrienols, the unsaturated forms of vitamin E. All of them have good antioxidant activity, and are under scrutiny as hypocholesterolemic agents [1]. It is a reasonably well-balanced food with functional properties that have shown to provide medicinal benefits. A recent study has suggested that optimization of germination conditions of *Amaranthus hypochondriacus* using response surface methodology, is an effective strategy to increase the total phenolics and total flavonoids for enhancing functionality with improved antioxidant activity [2]. The health benefits attributed include decreasing plasma cholesterol levels, stimulating the immune system, exerting an antitumor activity, reducing blood glucose levels and improving conditions of hypertension and anemia. In addition, it has been reported to have anti-allergic and antioxidant activities [3]. The Amaranth is gluten-free and hence easy to digest. The Amaranth grain is 90% digestible and because of its ease of digestion, it has traditionally been given to those recovering from illness or fasting period [4]. A seed of grain amaranth is on average composed of 13.1 to 21.0% of crude protein; 5.6 to 10.9% of crude fat; 48 to 69% of starch; 3.1 to 5.0% (14.2%) of dietary fibre and 2.5 to 4.4% of ash [5]. It is a terrific source of minerals like calcium, magnesium, and copper, a good source of zinc, potassium, and phosphorus. It helps to build strong bones and a muscle, aid hydration, boost energy, and is vital in thousands of processes. The genus Amaranth (L.) includes more than 60 species [6]. The species of the Amaranth that are used as a grain are *Amaranthus caudatus*, *Amaranthus cruentus*, and *Amaranthus hypochondriacus*.

2. Materials and methods
2.1 Sample collection
Dried grains of *Amaranthus hypochondriacus* were collected from a local market of Jammu and Kashmir in the month of October, 2013 and were authenticated as *Amaranthus hypochondriacus* (L.) under the reference number NISCAIR/RHMD/Consult/2014/2512/91.

Fig 1: Dried *Amaranthus hypochondriacus* (L.) grains

2. Materials and methods

2.1 Sample collection

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2.2 Extraction

The Amaranth grains were first ground to powder and then sieved. The flour so obtained was then stored in an air proof container for further use in experiments. To about 50 g of the Amaranth flour 200 ml of methanol, was added. The mixture was then incubated on an incubator shaker for 24 h and then filtered using Whatman filter paper 2. The solvent was evaporated in hot air oven at a temperature not exceeding 60 °C. The resulting extract so obtained was dark brownish in color. The extract was dissolved in DMSO to obtain 100mg in 1 ml stock and stored at 4 ºC till analysis.

2.3 Phytochemical analysis

2.3.1 Total phenolics

The estimation of the total phenolic content of the dried *Amaranthus hypochondriacus* grains was done using Folin – Ciocalteu method [7]. The results were expressed as Gallic Acid Equivalents (GAE, µg/mg of weight of extract). The absorbance of the standard (Gallic acid) and the extract of the grains were measured against DMSO blank spectrometrically at 765 nm. Each determination was performed in triplicate to avoid any error.

2.3.2 Total Flavonoids

Aluminium chloride colorimetric method [8] was used for the determination of the total flavonoid content. The optical density of the standard (catechin) and the sample extract was measured at 765 nm against DMSO blank, the total flavonoid content was expressed in µg of Catechin equivalents per mg of weight of extracts (CE, µg/mg of weight of extract).

2.3.3 Alkaloids and saponins content

The concentration of crude alkaloids and saponins was estimated using the methods already described by Harborne [9] and Obadoni & Ochuko [10] respectively. The results were calculated in percentage.

2.4 Determination of the antioxidant activity

The total antioxidant capacity of the extract was determined using ABTS radical scavenging activity and FRAP assay.

2.4.1 ABTS radical scavenging assay

The ABTS radicals are generated through a chemical oxidation reaction with potassium persulfate. The ABTS assay [2, 2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid] carried out was previously described by Re *et al*. (1999) [11]. The $ABTS^{+}$ radical cation was produced by mixing ABTS with potassium persulfate, the mixture was then kept in the dark at room temperature for 16 h. For the analysis, the reagent was diluted in ethanol until the absorption at 734 nm was 0.7± 0.02. A 20 µl of extract was mixed with 980 µl of ABTS reagent. The absorption was measured in a Hewlett- Packard spectrophotometer after 6 min of the addition of the sample. Each determination was performed in triplicates. Standard solution was made using ascorbic acid. The percentage of radical scavenging activity was calculated using the formula:

$$\% \text{ Scavenging activity} = \{A_{control} - A_{sample})/A_{control}] * 100$$

2.4.2 FRAP antioxidant Assay

The FRAP (Ferric Reducing Ability of Plasma) assay was carried out according to the method described by Benzie and Strain (1996) [12]. The FRAP reagent consisted of TPTZ in 40 mM HCl, $FeCl_3$ and sodium acetate (pH 3.6). FRAP reagent was freshly prepared. A 100 µl of extract solution containing 0.1 mg extract was mixed with 900 µl of FRAP reagent. The mixture was allowed to stand at 37 °C for 4 min, the absorbance at 593 nm was then determined against the blank. BHT dissolved in DMSO was used as a standard. FRAP values were calculated as mg of BHT equivalents/g extract from three determinations and were averaged.

2.5 Mineral Analysis

Minerals, trace elements and heavy metals in the Amaranth grains were determined by using Optima 2100 DV ICP-OES (Perkin-Elmer, USA), after prior mineralization in an Anton Paar Multiwave microwave digester (Anton Paar Ltd., Hertford, UK) as per Ref 956.52 AOAC, (2005) [13].

2.6 Antimicrobial activity

The agar well diffusion method [14] was used to test the antibacterial activity of extract against three gram – positive and one gram negative bacterial test pathogens. Extracts were reconstituted to a final concentration of 100 mg/ml. The Nutrient agar was inoculated by spreading 100 µl of the bacterial inoculums. Wells of 6 mm diameter were punched in the agar and 100 µl of extracts were loaded into the wells. The plates were incubated at 37 °C for 24 h. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition and reported on the scale of millimeters (mm).

2.7 Formulation of the fortified cookies

The fortified cookies were prepared using 7% Amaranth Flour, 7% refined wheat flour and 18% Oats using normal baking procedures. The cookies were then stored in air- tight containers and were evaluated later, for their nutraceutical properties.

2.8 Nutritional profiling of Cookies

The fortified cookies were evaluated for moisture content (AOAC, 1999) [15], ash content [13], protein content using a kjehldahl method (AOAC, 2005) [13], crude fiber content was evaluated (Ref. 978.10 of AOAC, 2005) [13] and crude fat content (AOAC method Ref. 2003.06) [16].

Total carbohydrates and energy content was calculated using formulae:

Total carbohydrates (%) = 100- [protein content %+Crude fat % + ash % + moisture%]

The calorific value in kilocalories (Kcal) = formula [(Protein +carbohydrate) *4 + (fat) *9]

2.9 Sensory evaluation of cookies

Sensory characteristics, namely colour, texture and crunchiness were rated using a 9 point hedonic scale.

Fig 2: Formulated Amaranth grain cookies

3. Results and discussion

3.1 Phytochemical analysis

The phytochemical analysis of the dried Amaranth grains was carried out and it was observed that the Amaranth grains are a moderate source of phytochemicals (Table 1). The Phytochemicals are biologically active compounds which provide health benefits and hence very attractive in the food industry. The Total Phenolics content of 8.45 GAE equivalents (µg GAE/mg sample) was estimated. The Total Flavonoids content of the reconstituted extract was estimated by the Aluminium chloride method and the results were expressed in µg of catechin equivalents (CE) per mg of dry weight of the extract based on the calibration curve of the standard. The total flavonoid content in the dried Amaranth seeds was estimated to be 6.29 µg of Catechin (CE) equivalents per mg.

Crude alkaloids and saponins in the Amaranth seed extract were estimated to be 5.57% and 0.06% respectively. These alkaloids in the diet consisting of the Amaranth grains may lead to healing of wounds, varicose ulcers, hemorrhoids, frost-bite and burn in herbal medicines. Saponins, on the other hand, will enhance the nutrient absorption, control blood cholesterol levels, bone health, cancer, and will build up the immune system. Saponins were obtained in negligible amounts in the sample.

Table 1: Phytochemical content of dry *Amaranthus hypochondriacus* grains

Analyte	Content
Total Phenolics	0.8459 mg GAE \100 mg extract
Total Flavonoids	0.629 mg CE \100 mg extract
Alkaloids	5.572%
Saponins	0.060%

3.2 Determination of antioxidant activity

3.2.1 ABTS scavenging assay

The total antioxidant activity of *Amaranthus hypochondriacus* L. grains were evaluated in accordance with decolourisation of ABTS to its radical cation ABTS+ as percentage inhibition using the % radical scavenging formula. IC_{50} value of methanolic extracts of the Amaranth grains determined by ABTS assay was found to be 19.1 mg/ml.

3.2.2 FRAP assay

In the Ferric Reducing Ability of Plasma (FRAP) assay, reduction of the ferric-tripyridyltriazine to the blue colour ferrous complex takes place which is measured at a wavelength of 593 nm. The intensity of the colour is related to the amount of antioxidant reductants in the samples. The FRAP activity was found to be very good in the sample (2.41 mg BHTE/ 100 mg).

3.3 Mineral Analysis

Mineral determination by ICP-OES revealed the presence of good amounts of essential minerals like Magnesium (848 µg/g), Calcium (519.3 µg/g), Phosphorus (330 µg/g) and Iron (65 µg/g). Magnesium helps in maintaining blood pressure, diabetes, asthma, heart attack and bone health. Calcium helps in improving bone health and dental health, as well as the prevention of colon cancer and the reduction of obesity. Phosphorous reduces muscle weakness, boosts brain function and optimizes body metabolism, whereas Iron plays a vital role in the formation of haemoglobin, which guarantees circulation of the blood and oxygenation of various organ systems, maintains body metabolism, muscle activity, anaemia, brain function, immunity, insomnia and the regulation of body temperature.

Table 2: Mineral content analysis of *Amaranthus hypochondriacus* grains

S no.	Mineral	Concentration (µg/g)
1	Sb	0.120
2	Cd	0.014
3	Ca	519.3
4	Cr	1.018
5	Co	0.085
6	Cu	2.846
7	Fe	65.41
8	Pb	0.307
9	Li	0.067
10	Mg	848.0
11	Mn	8.828
12	Mo	0.284
13	Ni	0.549
14	P	330
15	Se	0.038
16	Ti	0.876
17	Sn	0.245
18	Zn	28.93

3.4 Antibacterial Activity

The test bacterial pathogens used for the evaluation of antibacterial activity of the dried Amaranth grains were *Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis* and *Proteus mirabilis.* Agar well diffusion method was used to assess the antibacterial activity by measuring in terms of zone of inhibition. Inhibiting concentrations used for the sample was 100 mg/ml. No zone of inhibition was obtained in any of the four strains.

3.5 Nutritional profiling and sensory evaluation of Multigrain cookies

Nutritional studies of the fortified cookies have demonstrated its functionality as a nutraceutical (Table 3). The cookies were observed to be a very rich source of energy (434.72 Kcal). The ash content was estimated to be 2.11% and the moisture content was 5.02%. Low moisture content is desirable in terms of increasing the shelf life of the product. The total protein, carbohydrates and crude fibre content in the biscuits were estimated to be 8.85%, 64.37 and 3.88% by weight, respectively.

The parameters like taste, colour and the crunchiness of the cookies were evaluated for sensory analysis to understand the consumer acceptability of the product. The average score for taste was observed to be 9, indicating 'like extremely'. While for colour, it was estimated to be 7 indicating 'like moderately' and for the crunchiness of the biscuits an average of 9 was evaluated which indicated 'like very much'. These results depict a positive consumer acceptance of the Amaranth fortified cookies, which provide taste along with health benefits.

Table 3: Proximate analysis of fortified cookies

S. No.	Nutrients	Cookies
1	Energy (Kcal)	434.72
2	Ash	2.115%
3	Moisture	5.02%
4	Protein	8.85%
5	Fats	15.76%
6	Carbohydrates	64.37%
7	Dietary fiber	3.88%

4. Conclusion

The present study has contributed to understanding the potential of *Amaranthus hypochondriacus* grains as an excellent source of phytochemicals like phenolics, flavonoids, alkaloids and saponins. The various experimental analyses carried out on *Amaranthus hypochondriacus* grains revealed its potential as a good nutraceutical, thereby, offering various health benefits. The methanolic extract of the sample exhibited high antioxidant potential. The multigrain cookies fortified with the Amaranth grains indicated very good nutritional properties and mineral content and hence can be considered healthy for consumption. Based on the results presented here, it can be concluded that the methanolic extract of *Amaranthus hypochondriacus* is a rich source of bioactive compounds and thus offers several opportunities for the development functional foods and nutraceuticals.

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