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Ascorbic Acid and Antioxidant Activity in Germinating Horse Gram (*Macrotyloma uniflorum*) Seeds

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ABSTRACT

Health is functional efficiency of a living organism. It defines health in the broader sense of complete physical, mental, and social well-being. Approximately 36 million people die each year from non-communicable disease including cardiovascular disease, cancer, diabetes, and chronic lung disease. The aim of the present study was to estimate the concentration of ascorbic acid content and total antioxidant activity in germinating seeds of horse gram. The horse gram seeds were collected from the Ramanagara district, Karnataka state India. The ascorbic acid content was estimated by DNPH method and total antioxidant activity by DPPH method. The concentrations of ascorbic acid in moisture free and rehydrated horse gram seeds were found to be 17.25 mg and 20.97 mg per 100-gram seeds respectively. The present results showed 20.88, 30.08 and 30.27 mg at 12, 18 and 24 h / 100 g dry seeds of germination of horse gram seeds respectively. There was an increase of ascorbic acid up to 52.77 mg on the 4^{th} day of germination. The antioxidant activity was also determined and IC_{50} values of germinating horse gram seeds were estimated to 475, 285, 230, 345, 315 and 495µg /ml at 0, 6, 12, 18, 24 hrs of germination and moisture free of horse gram seeds respectively. Further, IC_{50} values from day 2 to day 7 were found 480, 595, 630, 895, 1005 and 895µg /ml in germinating seeds respectively. The ascorbic acid and total antioxidant activity were estimated and the results showed a good amount of ascorbic acid and total antioxidant activity in germinating seeds. Therefore, the germinated horse gram seeds are being used in the traditional diet as a beneficial source of food with very high nutritional value and support the concept of functional foods for maintaining good health. This information may useful to the food industry and an individual of the society.

INTRODUCTION

Food acts as nutraceuticals provide health benefits include protection, prevention, and treatment of diseases may be due to the mixture of cellular and biochemical interactions leads to the overall health benefits of an individual. The bioactive molecules are present in the horse gram is an important source to explore for drug designing to cure many diseases [15, 17]. Horse gram (*Macrotyloma uniflorum* L.) belongs to the family *Fabaceae* is an important legume food is due to high nutritional value as balance diet for rural and urban areas in developing countries. It is cultivated in Southern Asia, East and Northeast Africa, Australia and not explored its potential nutritional properties. The horse gram seeds showed quite a good amount of essential macronutrients and micronutrients (carotene, thiamin, riboflavin, niacin, ascorbic acid, and minerals), in addition, research data showed that anti-nutritional factors were affecting the bioavailability of nutrients. It is used as ethnomedicine, dietary food and lifestyle habits changes for curing many diseases includes kidney stones, asthma, heart diseases, diabetes and coronary heart disease due to free radical scavenging activity [3, 7, 10, 16, 21]. The post-prandial glucose levels due to impaired secretion of insulin can be controlled by the enzymes, the dietary fiber of horse gram seeds leads to the lowers blood glucose level and food processing method may influence on the glycemic index on diabetic populations [2, 8, 13, 24]. The free radicals are generated during food into energy conversion through metabolic functions. Oxidative stress appears in the system due to an imbalance between free radicals production and antioxidants status. The excessive free radicals may lead to stress-related diseases such as cataract, cancer, diabetes, Alzheimer's disease, Parkinson's disease, heart diseases aging and damage genetic material DNA. Free radicals may be protected in the system by the action of enzymes like catalase and superoxide dismutase and chemical compounds such as glutathione, ascorbic acid, and tocopherol [10, 16, 23, 25]. The free radicals may be prevented by the addition of antioxidants through the diet [16]. The grains are showing good antioxidant activity due to phenolic characteristic properties involved in many health benefits [12, 20]. The conventional processing technologies like drying, cooking, soaking, fermentation, germination lead to reduce the anti-nutritional factors. The soaking and germination conditions of 12-18hrs time reduce the phytic acid content and inhibitors of proteolytic enzymes which improves nutritional quality and also reported day1 to day 2 germination showed increase functional nutrients through reducing the anti-nutrients in legumes leads to enhance the bioavailability of divalent metals. Recent reports showed that there was a reduction in the total phenols content was noticed

might increase the activity of an enzyme polyphenol oxidase and catabolic enzymes are activated to hydrolyze nutrients include phenolic compounds during the germination process. A similar pattern was observed in the anti-oxidants with positive correlation due to phenolics and antioxidant activity [1, 17, 21,22]. Horse gram seeds extracts were showed the protection the condition of inflammation, anti-bacterial activity [9, 25]. Therefore, the present research study was to investigate the ascorbic acid content and antioxidant activity in dry and germinating horse gram seeds.

MATERIALS

Horse gram (*Macrotyloma uniflorum*) seeds were collected from the local market of Ramanagara District, Karnataka state, India. The chemicals used in the present study were the highest purity and analytical grade.

METHODS

Preparation of samples

The dry horse gram seeds were manually cleaned and finely powdered by using pestle and mortar. A sample of dry seeds and a fine powder was stored in a cool and dark place at room temperature for further analysis.

Germination conditions

The dry horse gram seeds were washed and soaked with water (1:9 w/v) for 12h and the excess water was drained. The soaked seeds were further germinated for different time periods at 0h, 6h, 12h, 18h, day1, day2, day3, day4, day5, day6 and day7 by traditional germination method wherein, the seeds were tied in a four folded moistened with white cotton cloth and were incubated at room temperature (17 to 27°C) in prevailing light and dark conditions during day and night periods respectively.

Extraction of ascorbic acid

The ascorbic acid extraction from the moisture fee, soaked and germinating seed samples were carried out under standard conditions by the method as described by Schaffert and Kingsley[18], Briefly, the moisture free, soaked and germinating samples were homogenized using a pestle and mortar to a fine paste by the addition of 5% trichloroacetic acid (TCA) and

the ascorbic acid was extracted further by the addition of 5% TCA (1:30 w/v) and sample were kept for 30 min and vortex for two minutes after every 10 min duration. The clear supernatant containing ascorbic acid was recovered by centrifugation for 15 min at 10000 rpm and 4°C.

Estimation of ascorbic acid

Preparation of standard graph: Ascorbic acid concentrations ranging from 8 to 40 µg /ml were pipette (0-1ml) in a series of test tubes and final volume was adjusted to 1 ml by the addition of 5% TCA solution. A 1ml of DNPH reagent was added to all the test tubes and the tubes were placed in a boiling water bath for 10 min. The test tubes were cooled to room temperature and then a 5 ml of ice-cold 85 % sulfuric acid was added slowly through the walls of the tubes. The contents of the test tubes were cooled and the color developed was read at 520 nm in a spectrophotometer (UV/Vis spectrophotometer, ELICO-SL 159). **Estimation of ascorbic acid:** The extracts of the moisture-free, soaked and germinating seeds were clarified by centrifugation at 10000 rpm for 10 min and 4°C. A 1 ml of suitably diluted clear supernatant of the extract was used for the estimation of ascorbic acid as shown above and mg ascorbic acid per 100 g seeds was represented as mg%. The results of the estimations are the representative of average values of three repeat experiments.

Total antioxidant activity

Extraction of antioxidants

The moisture free samples, soaked and germinating samples were used for extraction of antioxidants through reflux condensation with the solvent system (1%HCl-methanol) for 3h. After reflux condensation samples were collected and removal of solvent using hot air oven. Refluxed extract samples were used for the antioxidant activity and remaining samples were stored at 4^{o}C for further analysis.

Total antioxidant activity

The total antioxidant activity in the sample was carried by the method of Brand-Willams W [4]. Briefly, based on the reducing power of antioxidants by measuring the deep violet color of DPPH to colorless by receiving hydrogen through free radical scavenging ability was read absorbance at 517 by using a spectrophotometer. The refluxed extract sample was weighed

into clean test tubes (upto10mg), 2.5ml of 1% HCl-methanol was added and dissolved completely then make up the final volume to 5ml by addition 2.5ml distilled water. The samples were used for the estimation 0.1, 0.2, 0.3, 0.4 and 0.5ml in the different test tubes after thorough mixing then the volume makes up to 960 µl by the addition of 50% methanol and 140µl of DPPH was added to all the tubes and incubates at 37°C for 30 min. After incubation samples were scanned from 190nm-840nm (absorbance recorded at 517 nm) in Uv-VIS spectrophotometer (Thermo-Scientific Model Evolution 220). The results of antioxidants were calculated by using formula.

$$\% \text{ Inhibition} = [(A_0-A_1)/A_0] \text{ X } 100$$

Where, A_0 - Control absorbance, A_1 - Extract Standard absorbance

Lower the value of absorbance is the higher radical scavenging activity. The antioxidant activity is expressed as IC_{50} value (µg/ml) of extracts inhibits the DPPH radicals by 50 %.

RESULTS

Horse gram (*Macrotyloma uniflorum*) seeds were collected from the local market of Ramanagara District, Karnataka state, India. The seeds were manually cleaned and fine powdered was stored in a cool and dark place at room temperature. For germination, seeds were washed and soaked with water for 12h and the excess water was drained. The soaked seeds were further germinated for different time periods at 0h, 6h, 12h, 18h, day1, day2, day3, day4, day5, day6 and day7 by traditional germination technique and incubated at room temperature (17 to 27°C) in prevailing light and dark conditions during day and night periods respectively.

Ascorbic acid: Ascorbic acid was extracted with 5% trichloroacetic acid and estimated by DNPH method in soaking and germinating horse gram seeds. The construction of standard ascorbic acid linearity graph was carried out at concentrations ranging from 0 to 40 µg /ml. The result of the standard ascorbic acid was plotted and a graph of absorption v/s concentration of ascorbic acid showing linear regression, r^2 - 0.995. The ascorbic acid concentration in moisture free and rehydrated seeds was found to be 17.25 mg and 20.97 mg /100g respectively. The horse gram seeds were processed by germination over a period of 7 days as described in material and methods. The present study results showed ascorbic acid content found to be 20.80mg, 20.88 mg, 30.08 mg and 30.27 mg /100g at 6, 12, 18 and 24 h

of germination time respectively (Table 1 and Figure 1a). The changes in the ascorbic acid content were further monitored calorimetrically in the extracts of germinating seeds after germination for 2 to 7 days and were found to be 45.08, 46.94, 52.77, 38.05, 34.52 and 30.75 mg /100g seeds respectively (Table 1 and Figure 1a).

Table 1: Ascorbic acid (AsA), IC_{50} value and Inhibition of hydroxyl radicals scavenging activity.

Time	0h	6h	12h	18h	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Dry
AsA (mg/100g)	20.97	20.80	20.88	30.08	30.27	45.08	46.94	52.77	38.05	34.52	30.75	17.25
IC_{50} value (µg/ml)	285	230	345	315	495	480	595	630	895	1005	895	475
Inhibition (%)	72.9	75.6	69.9	71.9	65.3	79.5	79.5	72.4	60.5	66.8	73.2	75.3

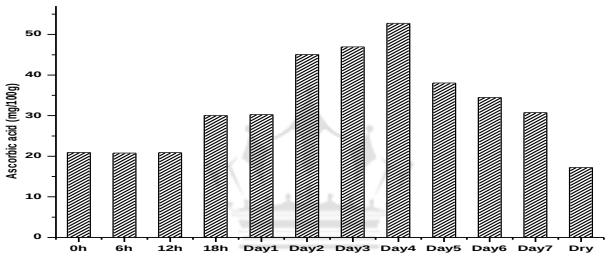


Figure 1a: Ascorbic acid content (mg / 100 g).

Antioxidant activity and inhibition of hydroxyl radicals scavenging activity: The concentration of antioxidant activity in the germinating seeds as shown in Table 1 and Figure 1b. The IC_{50} values of germinating horse gram seeds were estimated to be 285, 230, 345, 315, 495 and 475 µg /ml at 0, 6, 12, 18, 24h and moisture free sample. The changes in the antioxidant content further monitored in the extracts of germinating seeds after germination for 2 to 7 days and IC_{50} values were found to be 480, 595, 630, 895, 1005, 895µg /ml seeds respectively on day 2 to day 7 (Table 1 and Figure 1b). The inhibition of hydroxyl radicals scavenging activity in germinating horse gram seeds was found to be 72.9, 75.6, 69.9, 71.9, 65.3 and 75.3 % at 0, 6, 12, 18, 24h and moisture free sample. The inhibition of hydroxyl radicals scavenging activity changes was a monitor in the extracts of germinating seeds after germination for 2 to 7 days and inhibition was found to be 79.5, 79.5, 72.4, 60.5, 66.8, 73.2 % respectively from day 2 to day 7 (Table 1, Figure 1b and 1c).

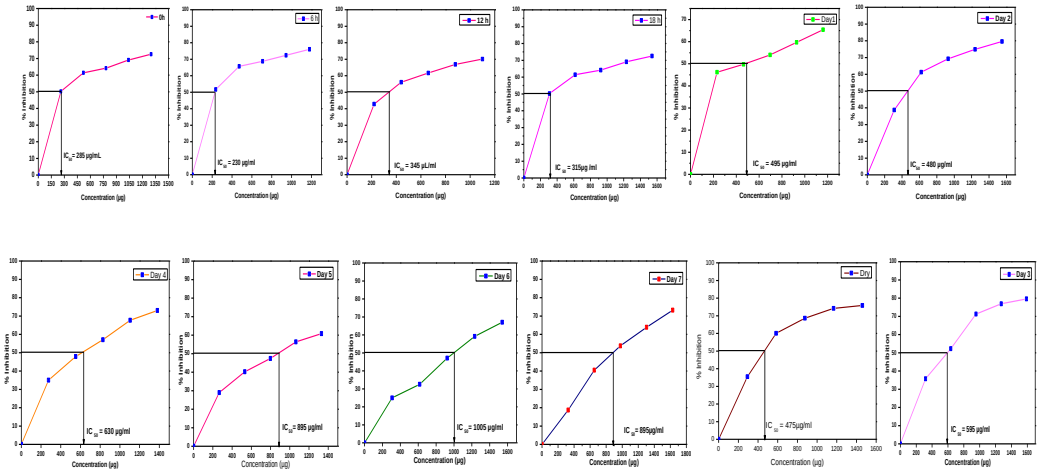


Figure 1b: Inhibition of hydroxyl radicals scavenging activity

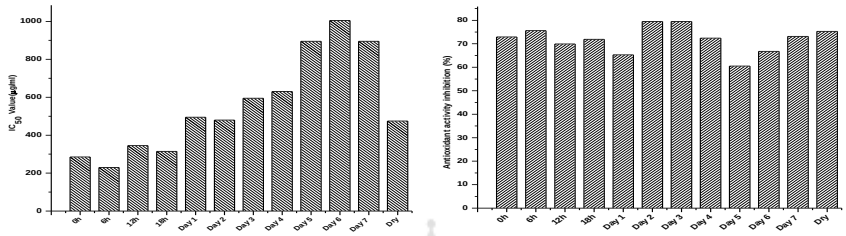


Figure 1c: IC_{50} value and inhibition of antioxidant activity (%)

DISCUSSION

The free radicals generated from the oxidation of macromolecules leads to degenerative diseases. Polyphenols and ascorbic acid may prevent degenerative diseases through sequester free radicals are due to antioxidant properties [5]. The recommended dietary intake of ascorbic acid may be based on the amount required for the biosynthesis and maintenance. Low serum levels of ascorbic acid may have serious health implications and are particularly relevant to the onset of age-related problems and degenerative diseases. Legumes are good sources of proteins, minerals and fair sources of vitamins. The nutritional contents of some pulses have been reported to increase by processing technique such as germination improves the texture, palatability, antioxidant capacity, vitamins, and bioavailability of minerals. In addition, protein inhibitor also presents that can reduce by the traditional method of the germination process. Due to antioxidant properties of phytochemicals can prevent many diseases like diabetes, cancer, renal stones and heart diseases Appreciable amount of phytochemicals such as phenolic compounds are *p*-coumaric acid, *p*-hydroxybenzoic acid accumulated and showed antioxidant activity resulted in the quenching of free radicals. The

recent research data showed the moisture content was ranged from 9 to 11 % and ash content ranged from 2.7 to 3.4 %. The dietary fiber 15 % of insoluble fiber and soluble fiber content 1.4 %. The fat content was ranged from 0.5 to 1.4 %. The protein content was ranged from 18 to 25 %. The carbohydrate content was range from 52 to 67%, starch 36 %, 85 % of digestible and 14% of resistant starch [3]. The horse gram seeds were processed by germination technique as described in material and methods. We have estimated the ascorbic acid in dry (moisture free), rehydrated and germinating seeds of horse gram. However, processing by simple germination has brought about appreciable changes in the ascorbic acid in a time-dependent manner. Thippeswamy T G et al., [19] showed ascorbic acid content in mung bean seeds of germination raw dry seeds were showed 27.25 mg/100g increased up to 114.23 mg/100g germinating seeds. 89.52 mg% of ascorbic acid was showed at 24h of germination time. Ranasinghe RDLS *et al*., [13] revealed ascorbic acid content was 0.7 % in horse gram seeds but in the present study showed that 17.25 mg% ascorbic acid in seed flour. Vanshika Handa *et al*., [22] showed the ascorbic acid content 4.0, 5.33, 8.00 and 8.67 mg /100g at 0h, 6h, and 18 respectively. The ascorbic acid content showed in germination time was 8.67, 9.33 and 10.0 mg/100g at 0h, 24h and 48 h respectively. The present study showed high ascorbic acid content of germinating horse gram seeds were estimated to be 20.97, 30.27 17 and 45.08 mg /100g at 0, 24h and 48h respectively.

The concentrations of ascorbic acid in dry (moisture free) and rehydrated horse gram seeds were found to be 17.25 and 20.97 mg /100g respectively (Table1 and Figure1a). The ascorbic acid content of germinating horse gram seeds was estimated to be 20.80, 20.88, 30.08 and 30.27mg /100g at 6, 12, 18 and 24 h respectively in germination seeds. The changes in the ascorbic acid content were further monitored calorimetrically in the extracts of germinating seeds after germination for 2 to 7 days and were found to be 45.08, 46.94, 52.77, 38.05, 34.52 and 30.75 mg /100g seeds respectively (Table1 and Figure1a). The ascorbic acid enhancement during germination may be due to the activation of enzyme L-Galactono-γ-lactone dehydrogenase in the formation of ascorbic acid from L-galactono-1, 4-lactone [22]. Laxmi H. Gupta et al., [8] reported the management of post-prandial glucose levels due to impaired secretion of insulin in the blood by supplementation of horse gram extracts to control diabetes mellitus through α- amylase and α- glucosidase inhibitors. Vrinda Rajagopal *et al*., [25] clearly suggest an effect of pro-inflammatory activity and positive change in the NOS enzyme after 21 and 60-day experiments indicating cytokines activity in epithelial cells. The study of anticancer using horse gram seeds against human osteosarcoma cell line with

ethanol and methanol extracts [6]. Perumal Siddaraju *et al*., [10] showed free radical scavenging activity in processed horse gram seeds against the ascorbic acid standard. The results showed the black variety of horse gram seeds were the highest scavenging activity. Ashok Kumar Tiwari *et al*., [2] report showed that decrease the total polyphenols, flavonoids during germination may be due to the removal of the seed coat during germination compared to the cotyledons. It has been identified insulin-like proteins during germination so that the diabetic individuals prefer to consumption of germinating seeds and antioxidant activity in the course of germination may increase in some legumes. Abiraami Valli and Uma Gowrie [1] reported anti-oxidant activity in fresh seeds of IC_{50} value were 229 and 251 µg /ml in methanol and aqueous extract respectively. In the dried seed flour showed 244 and 266 µg/ml in methanol and aqueous extract respectively by hydrogen peroxide scavenging activity. The present study showed high IC_{50} value 475 µg /ml in the acid-methanol extract of the dry sample by hydroxyl radicals scavenging activity. Ramesh *et al*., [12] showed that the dry seeds were showed IC_{50} values of germination seeds 3.66 and 4.225 mg/ml respectively and germination result showed high compared to dry seeds in methanol extract. Varicola Karuna Sree *et al*., [23] results showed the IC_{50} value of standard was 3.95 µg/ ml and methanol extract was found to be 3.86 µg/ ml.

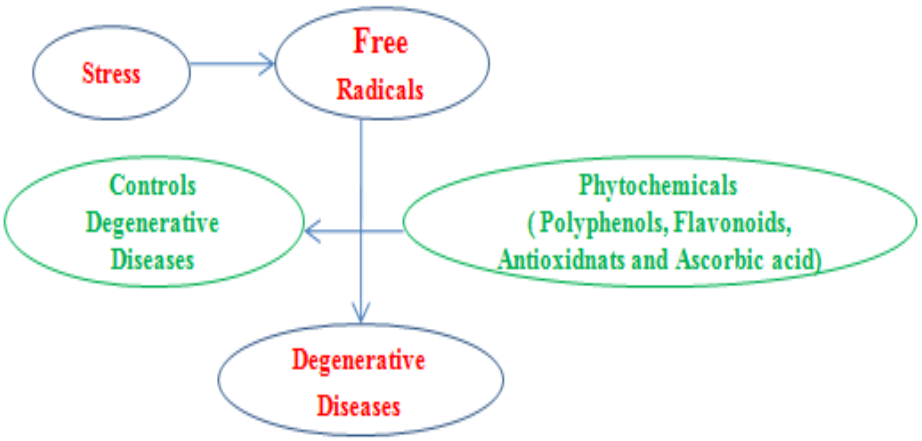
Present results showed that the antioxidant activity in germinating seeds of horse gram showed the IC_{50} values were estimated to 475, 280, 230, 345, 315 and 495µg at dry, 0, 6, 12, 18 and 24 h of germination of horse gram seeds respectively. The changes in the antioxidant content further monitored in the extracts of germinating seeds after germination for 2 to 7 days and IC_{50} values were found to be 480, 595, 630, 895, 1005, 895µg /ml seeds respectively. The present study indicates antioxidant activity in an acid-methanol extract of horse gram seeds is showing high at 18h of germination time. The antioxidants were extracted with acid-methanol by reflux condenser for 3h.

The inhibition of hydroxyl radicals scavenging activity was calculated and compared with the earlier studies. Gayatri Chakraborty *et al*., [6] showed the inhibition of scavenging activity was showed as LD_{50} 57.73 µg/ ml ascorbic acid, 65.35, 62.99, 68.7, 61.81 µg/ml in various horse gram seeds samples. The concentration of ethanol or methanol at 200 µg/ml extract showed 82 % and 74 % cell viability and also proved that the horse gram anticancer activity against B16F10 and B16BL6 mouse melanoma cell line[6]. The inhibition of scavenging activity was showed maximum 79.5 % by DPPH method when compared to the report of

Ravishankar et al., [14] where scavenging of inhibition was 62.03 % by hydroxyl method in ethanol extract. Vanshika Handa et al., [22] showed the anti-oxidant activity inhibition of hydroxyl radicals scavenging activity was 89.55, 87.94, 87.11 and 85.18% at 0h, 6h, and 18h respectively. Petchiammal C and Waheeta Hopper [11] report showed inhibition of hydroxyl radicals scavenging activity in black horse gram was 53.3% and brown horse gram was 56.1 %. The ethanol extracts of horse gram seeds showed antibacterial activity compared to aqueous extracts [9].In the present study reveals inhibition at 500 µg/ml were showed 72.9, 75.6, 69.89, 71.9, 65.32, 79.5, 79.5, 72.4, 60.5, 66.8, 73.2 and 75.3% at 0h, 6h, 12h ,18h, day1, day2, day3, day3, day 4, day5, day6, day7 and dry (moisture free) respectively in acid-methanol extract (Table1 and Figure1c). The present investigation results showed an appreciable amount of ascorbic acid and total antioxidant in horse gram seeds in dry and germinating seeds, in turn, to control or prevent many degenerative diseases.

CONCLUSION

Horse gram seeds were used as therapeutic bioactive compounds along with excellent nutritional quality makes it a whole food that should be added to diet on regular basis. It has been hypothesized to be because they contain phytochemicals that combat oxidative stress in the body by helping to maintain a balance between oxidants and antioxidants. The ascorbic acid and total antioxidant activity in germinating seeds were estimated and the results showed a good amount of ascorbic acid and total antioxidant activity to our daily-recommended allowance to maintain good health. The germinated horse gram seeds are being used in the traditional diet as a beneficial source of food with very high nutritional value and support the concept of functional foods. This information may useful to the food industry and an individual of the society.



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