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Pharmacokinetic study of amaranth extract in healthy human subjects-A randomized trial

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Running title: Pharmacokinetic study of amaranth extract

Abstract

Objective: Nitric oxide (NO) is one of the most important signaling molecules produced within the body. Continuous generation of NO is essential for the integrity of the cardiovascular system. The objective of the present study was to assess whether oral intake of a nitrate (NO_3^-) rich dietary supplement (amaranth extract) is able to increase NO_3^- and nitrite (NO_2^-) levels in blood plasma and saliva of healthy adults.

Methods: In the present study, bioavailability and pharmacokinetics of NO_3^- and NO_2^- from amaranth extract (2 g as single dose) was studied in 16 healthy subjects and compared with placebo in a crossover design. The NO_3^- and NO_2^- levels in plasma as well as saliva were measured up to 24 h.

Results: After administration of amaranth extract, the NO_3^- level in plasma as well as saliva were found to be significantly ($p<0.001$) higher than that were found in the placebo group. The NO_2^- level in plasma was slightly higher ($p<0.05$) in amaranth group (test group) as compared to that in the placebo group whereas saliva NO_2^- level was significantly high ($p<0.001$) in amaranth extract treated group than placebo group.

Conclusions: These results clearly indicate that a single oral dose of amaranth extract is able to increase the NO_3^- and NO_2^- levels in the body for at least eight hours. The increase in NO_3^- and NO_2^- levels can help in increasing the overall performance of people involved in vigorous physical activities or sports.

Keywords: Nitrate; Oxystorm; Amaranth extract; Nitric oxide; Nitrite; Red spinach

Introduction

A diet rich in vegetables has been described beneficial for longevity and overall health. The positive effects of vegetables may be attributed, in part, to inorganic nitrate (NO_3^-) which is present abundantly in green leafy vegetables [1,2]. To elicit any biological effects NO_3^- are likely to be converted to the nitrite (NO_2^-) ion in the mouth via facultative anaerobic bacteria on the surface of the tongue [3]. When swallowed, NO_2^- is further converted into nitric oxide (NO). The reduction of NO_2^- to NO and other reactive nitrogen intermediates are facilitated in hypoxia [4]. The production of NO via nitric oxide synthase (NOS) is impaired in hypoxia and, thus, it has been proposed that the NO_3^-- NO_2^--NO pathway represents a complementary system for NO generation across a wide range of redox states [5]. NO is an essential physiological signaling molecule with numerous functions in the body, including the regulation of blood flow, muscle contractility, glucose and calcium homeostasis, and mitochondrial respiration and biogenesis [6,7].

There is now substantial evidence that dietary NO_3^- supplementation can significantly increase the NO_2^- level and reduce resting blood pressure in young adults [8-11]. Moreover, dietary NO_3^- supplementation may have positive effects upon the physiological response to exercise [8,12]. Supplementation with $NaNO_3$ [12] or beetroot juice [13] resulted in a significant reduction in oxygen uptake during submaximal cycling. In a recent placebo controlled study, it is reported that beetroot juice supplementation significantly reduced the O_2 cost of treadmill walking and improved exercise tolerance in healthy young adults [14]. These results are remarkable because the oxygen uptake and work rate relationship have traditionally been considered to be independent of age, health status, and aerobic fitness [15]. The reduction in the O_2 cost of moderate intensity exercise following dietary NO_3^- supplementation may be a result of a reduced ATP cost of muscle force production [8] and/or enhanced mitochondrial efficiency

[16]. In a study by Stokes et al, dietary supplementation of NO_2^- and NO_3^- in mice has shown to reverse endothelial dysfunction, suppresses microvascular inflammation, reduces level of C-reactive protein in mice subjected to a high-cholesterol diet [17].

The availability of the NOS substrate L-arginine, and especially the NOS cofactor tetrahydrobiopterin, is lower in older age [18], which together with lower NO_2^-, a sensitive marker of NOS activity, suggests that NO synthesis through the NOS-NO pathway might be impaired with the process of aging [19]. In addition, superoxide (O_2^-) production is increased with aging, which would lower NO bioavailability, given the rapid reaction between (O_2^-) and NO to form peroxynitrite [20]. Given the positive association between NO and vascular health, these aging related perturbations to NO metabolism might contribute towards the endothelial dysfunction [21] and arterial hypertension [22] that develop with old age. Therefore, it is feasible that dietary NO_3^- supplementation might enhance NO bioavailability and vascular function in older adults.

Leafy vegetables and roots/rhizomes of some edible plants are rich source of dietary NO_3^-. Amaranth (red spinach) is one of such plants popularly grown as leafy vegetable in tropical regions of the world including Africa, India, Bangladesh, Sri Lanka and the Caribbean. It is also grown as leafy vegetable through South-East Asia and Latin America. Leaves as well as grains/seeds of amaranth are edible and contains large amount of NO_3^- along with other nutrients [23]. Amaranth leaves are also an excellent source of carotenoids, iron, calcium, ascorbic acid and proteins [24]. Consuming leafy vegetables in large quantities as a daily diet may not be enough to produce significant levels of NO_3^- and NO_2^- in blood and to get clinical benefits. In a recent human clinical study in older adults, plasma NO_3^- and NO_2^- were increased by a high NO_3^- supplement, but not by high NO_3^- foods [25].

The purpose of the present study, therefore, was to assess whether oral intake of a NO_3^- rich dietary supplement (amaranth extract) is able to increase NO_3^- and NO_2^- levels in blood plasma and saliva of healthy adults. The study was designed as placebo controlled, randomized, crossover study in sixteen healthy subjects.

Methods and materials

Study drugs

2 g of amaranth extract (Arjuna Natural Extracts Ltd., Aluva, Kerala, India) was used for test, whereas 2 g of glucose (99.4% D-glucose) was used as placebo.

Subjects

Twenty three subjects were screened and out of them, sixteen healthy adult male subjects (age : 18-40 years) meeting inclusion criteria were selected for the study. Study protocol was explained to all the subjects and the subjects willingly signed a consent form to participate in the trial. The study was approved by ethics committee of Good Society for Ethical Research, Delhi (GSER/ND-2014/AP/03) and registered with Clinical Trials Registry-India (CTRI registration no.: CTRI/2014/11/005192).

Subjects between the ages of 18 and 40 years (both inclusive), weighing at least 50 kg, with Body Mass Index (BMI) in the range 18.5-30.0 kg/m^2 and were able and ready to provide written informed consent were the inclusion criteria. Subjects must be of normal health as determined by medical history and physical examination, ECG, Chest X-ray (PA View) and laboratory tests were performed 21 days prior to the commencement of the study.

Subjects were excluded if they were incapable of understanding the informed consent process or not ready to sign informed consent, subjects on current use of organic nitrates, subjects with significant history of hypersensitivity to leafy vegetable extract or amaranth, subjects with signs or history of significant gastrointestinal, liver or kidney disease, significantly low or high blood pressure or any conditions known to interfere (e.g. people taking any medicines or food supplements) with the absorption, distribution, metabolism or excretion of amaranth extract. Subjects who had difficulty in donating blood and subjects with positive breath alcohol analysis or urine drug screen of abuse were also excluded.

Design and dietary interventions

This study was a two arm randomized crossover design consisting of amaranth extract (test product) and control (placebo). Study participants were randomly assigned to one of the arm and then crossed over after two weeks washout period. This ensured that all participants received each of the two interventions.

Subjects checked-in to the clinical facility at least 12-14 h prior to the test sample administration.. Subjects were not allowed to eat anything for 10 hours before taking the baseline venous blood sample.A single oral dose of either 2 g amaranth extract powder (test product) or 2 g glucose powder (placebo) dissolved in 300 ml lukewarm distilled water was administered to each subject at room temperature in sitting posture, in each period.

Post-dose blood samples (6 ml at each time) were collected at 00.25, 00.50, 00.75, 01.00, 01.50, 02.00, 02.50, 03.00, 04.00, 05.00, 06.00, 08.00 and 24.00 h in blood collecting vials containing Lithium heparin as anticoagulant. The blood samples were centrifuged at 2800 g and

plasma was carefully drawn and stored at -80°C until analysis. Saliva samples (4 ml at each time) were also collected in cryovials at the same time and stored at -80°C until analysis.

Food was restricted up to 6 h post dosing with test sample. The limitation of drinking water was maintained for 2h (1h before dosing and 1h after dosing) except during the administration of the test samples. Mid day snack, evening snack and dinner were provided at 6, 9 and 12 h post dose respectively in each period of the study. Cleaning teeth, tongue, and make use of oral mouth wash were not permitted on the day of study until the last sample was collected.

Nitrate and nitrite analysis

The plasma and saliva samples were processed and analyzed for NO_3^- and NO_2^- content by a validated UPLC method (under publication). In brief, WATERS AQUITY H Class UPLC system attached with column compartment, UPLC Sample Manager FTN, liquid chromatograph (LC) with quaternary solvent manager and detector (PDA eλ detector; 200-600 nm) were used. The column was ACQUITY UPLC BEH C18 having dimension 50 x 2.1 mm and particle size 1.7 µm. AQT, Waters Empower 2 was used as UPLC software. Gradient programming was used with a flow rate of 0.1-0.2 ml/min. Three mobile phases were used for elution. Mobile phase A was prepared by dissolving 1.4 gm tetrabutyl ammonium hydroxide in HPLC grade water and volume was made up to 1000 ml; pH of the solution was adjusted to 2.5 with concentrated sulfuric acid and filtered through 0.2 µ filter. HPLC grade acetonitrile was used as mobile phase B whereas methanol was used as mobile phase C. Injection volume was 2 µL in each case.

Accurately weighed 1.5–2.0 ml of plasma or saliva sample was deproteinized using acetonitrile and centrifuged at 19700 g at 5°C for 15 min. The supernatants were filtered through

158 0.2 micron filter and used in the UPLC for direct injection to analyze NO_3^- at 222 nm. For the
159 quantification of NO_2^-, a part of the supernatant liquid was derivatized with Griess reagent,
160 injected into the UPLC and the chromatogram was monitored at 520 nm. Griess reagent
161 comprises of sulfanilamide (Griess A) and 1-naphthyl ethylenediamine (Griess B). This reagent
162 converts NO_2^- into deep purple azo compound which is detectable by PDA detector and
163 concentration of NO_2^- can be determined.

164 *Pharmacokinetic and statistical analysis*

165 The pharmacokinetic analysis was performed using non-compartment model by WinNonlin
166 version 5.3 and parameters like C_{max}, T_{max} and AUC were calculated. The data was analyzed for
167 significance by one way ANOVA.

168 **Results**

169 Sixteen subjects were recruited for the study. All the subjects completed the period one study
170 whereas one dropped out in the second period of study for reasons best known to him. Ingestion
171 of amaranth extract/glucose powder was tolerated well by all subjects. None of the subjects
172 reported any discomfort or side effects.

173 *Plasma nitrate and nitrite*

174 The mean plasma NO_3^- level after administration of amaranth extract and placebo are presented
175 in Fig. 1. There was no significant difference between treatments in the baseline (i.e., zero hour)
176 plasma NO_3^- concentrations. After administration of amaranth extract, NO_3^- level increased
177 significantly and the maximum concentration (252.56 ± 8.60 μmol/L) was observed at 1 h.
178 Moreover, the level of NO_3^- in plasma remained significantly elevated ($p<0.001$) for at least

eight hours post-dose. In the case of placebo, the mean NO_3^- level did not increase and remained almost the same as it was observed at 0 h.

The plasma NO_2^- level also increased after the administration of amaranth extract (Fig. 2). The maximum NO_2^- level after ingestion of amaranth extract was 0.56 ± 0.06 μmol/L at 0.5 hr. The placebo was not able to increase the mean NO_2^- level in plasma significantly ($p>0.05$) as compared to baseline value.

Saliva nitrate and nitrite

Since about 30% of absorbed NO_3^- secretes into the saliva and there it reduces into NO_2^- by oral facultative bacteria, thus saliva was also analyzed for the presence of NO_3^- and NO_2^-. The mean level of NO_3^- in saliva after administration of amaranth extract and placebo are presented in Fig. 3. Initially, at baseline there was no significant difference between the concentration of NO_3^- in the saliva of the test group and the placebo group. After administration of amaranth extract, NO_3^- level in saliva increased many folds and the maximum concentration (3126.68 ± 331.11 μmol/L) was observed at 2.5 h. Similar to the level of NO_3^- in plasma, the level of NO_3^- in saliva also remained significantly elevated ($p<0.001$) for at least 8 h post-dose. In the case of placebo, the mean NO_3^- level in saliva did not increase and remained almost the same as it was observed at baseline.

After the administration of amaranth extract, there has been a significant increase in the concentration level of NO_2^- in the saliva ($p<0.001$) as compared to the baseline value (Fig. 4). The maximum NO_2^- level in the saliva after ingestion of amaranth extract was 1080.51 ± 98.89 μmol/L at 0.75 h. The placebo was not able to increase the mean NO_2^- level in saliva significantly ($p>0.05$) as compared to the baseline value.

Pharmacokinetic parameters

Pharmacokinetic parameters for NO_3^- and NO_2^- in plasma for the amaranth extract and placebo groups are presented in Table 1. AUC_{0-t} for plasma NO_3^- in amaranth extract and placebo group was 3095.64 ± 179.58 and 1541.02 ± 102.76 μmol.h/ml, respectively, which is highly significant ($p<0.001$). C_{max} was found to be 252.56 ± 8.60 and 69.34 ± 6.49 μmol/L respectively which is also highly significant ($p<0.001$). T_{max} of plasma NO_3^- of the two groups was also significantly different ($p<0.01$). C_{max} of plasma NO_2^- in the test group (0.56 ± 0.06 μmol/L) was significantly different ($p<0.01$) from that of the placebo group (0.36 ± 0.04 μmol/L). AUC_{0-t} and T_{max} of plasma NO_2^- of the test group were not significantly different ($p>0.05$) from that of placebo group.

Table 2 depicts the pharmacokinetic parameters for NO_3^- and NO_2^- in saliva. AUC_{0-t} of NO_3^- of the test group and the placebo group were 24017.47 ± 946.50 and 9129.54 ± 492.50 μmol.h/ml, showing highly significant difference ($p<0.001$) between the groups. In the same way, difference in C_{max} (3126.68 ± 331.11 for the test group and 519.77 ± 51.58 μmol/L for placebo group) was also significantly high ($p<0.001$) between the two groups. The difference of T_{max} of saliva NO_3^- between the two groups was not significantly different .

In contrast to plasma, the AUC_{0-t} (12035.16 ± 620.10 and 4992.94 ± 297.06 μmol.h/ml for the test group and the placebo group, respectively) of NO_2^- in the saliva of the two groups shows a highly significant difference ($p<0.001$). C_{max} of NO_2^- in the saliva of the two groups is also showing a highly significant difference ($p<0.001$) whereas T_{max} was not significantly different.

Discussion

Nitric oxide (NO) is one of the most important signaling molecules produced within the body. The loss of NO generation because of endothelial dysfunction is one of the major causes of cardio vascular diseases [26]. Continuous generation of NO is essential for the integrity of the cardiovascular system [27]. The first pathway for the endogenous production of NO is through the oxidation of the guanidino nitrogen group of L-arginine (a semi-essential amino acid) by a group of enzymes called nitric oxide synthase (NOS) localized to the vascular endothelium [28]. For many years, scientists and physicians have investigated L-arginine supplementation as a means to enhance NO production. However, patients with endothelial dysfunction, by definition, are unable to convert L-arginine to NO; and therefore, this strategy has failed in clinical trials [29].

Apart from patients suffering from endothelial dysfunction, the sports person or people, doing excessive exercise/physical work, require more NO especially during hypoxia. In this study, the amaranth extract is found to enhance significantly the concentration of NO_3^- in the plasma within 30 min of intake and it reached the maximum in 1 h. It is well known that large amount of NO_3^- secretes in saliva where part of it converts into NO_2^- and then after mixing with stomach acid further converts into nitrous acid and finally to many nitrogen species including NO. In this study, the concentration of NO_3^- in saliva reached the maximum in 2.5 h which is significantly higher than T_{max} of NO_3^- concentration in plasma, which proves the earlier findings. Since the anaerobic oral facultative bacteria in mouth converts NO_3^- into NO_2^-, after administration of amaranth extract, concentration of NO_2^- in saliva was found to be significantly high ($p<0.001$) as compared to the placebo group. The concentration of NO_2^- in saliva reached the maximum in less than one hour which can be correlated with NO_3^- level in plasma (T_{max} = 1 h). Total NO concentration is commonly determined as a sum of NO_3^- and NO_2^- concentrations

[30]. Since NO_3^- and NO_2^- are two major metabolites of NO, in this study, an increase in NO_3^- and NO_2^- levels in plasma as well as saliva gives an indication of enhanced NO level in the body. The NO_2^- level in plasma was not continuously high for the whole duration of the study. At times ups and downs were observed. $NO_3^-$is getting converted into NO_2^- in the oral cavity with the help of facultative bacteria present in mouth may be the rate limiting step and may be the reason for fluctuations (ups and downs) in NO_2^- levesl in plasma.

In this study, there were no adverse events or any discomfort reported by any of the participants, this study also confirms the tolerability and safety of amaranth extract at the tested dosage (2 g) in human subjects. The pre and post study clinical parameters were not significantly different for all the subjects.

In a recent study on mice by Carlstrom et al, it was reported that dietary inorganic NO_3^- reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice [31]. This proof of concept has now been extended to humans supplemented with dietary sources of NO_3^-. Dietary NO_3^- has also been shown to reduce blood pressure, inhibit platelet aggregation, and restore endothelial function [9,11,32]. Increased NO bioavailability might also enhance brain blood flow and cognitive function. In addition to brain shrinkage in senescence, the capacity of the brain to produce ATP via oxidative phosphorylation decreases and, in combination with chronic ischemia of white matter, this results in a decline of cognitive function [33]. Furthermore, age-related mitochondrial dysfunction has been associated with the neuronal loss, which is a feature of neurodegenerative diseases. Recent studies suggest that NO plays a key role in cerebral vasodilation and blood flow, neurotransmission, and the coupling of neural activity to local cerebral blood flow [34]. Therefore, dietary NO_3^- supplementation may have the potential to modify cerebrovascular physiology and enhance cognitive function.

It is clearly emerging that the L-arginine pathway becomes dysfunctional with age, and also this pathway is not enough to supply the huge demand of NO by sports persons or the people doing vigorous exercise, thus a need arises for a backup system to compensate. Amaranth extract can be a useful supplement for the production of NO to prevent cardio vascular diseases in case endothelial dysfunctions .It can be equally useful for the sports persons or before any strenuous physical activity.

Conclusion

The results of this study clearly indicate that a single oral dose of amaranth extract is able to increase the levels of NO_3^- and NO_2^- in the body for at least eight hours. The increase in NO_3^- and NO_2^- levels can help in increasing the overall performance of people involved in vigorous physical activities or sports. Since deficiency of NO is one of the reasons for endothelial dysfunction and disorders related to aging, the amaranth extract may also be beneficial for the aged..

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Conflict of Interest

None

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401 **Figure legends:**

402 **Fig. 1 - Plasma nitrate (NO_3^-) level after administration of amaranth extract and placebo**
403 (highly significant difference ($p<0.001$) between amaranth and placebo group at all the time
404 points except 0 h and 24 h)

405 **Fig. 2 - Plasma nitrite (NO_2^-) level after administration of amaranth extract and placebo**
406 (highly significant difference ($p<0.001$) between amaranth and placebo group at 0.5, 1.5 and 3 h.
407 Significant difference ($p<0.01$) at 0.75, 2, 2.5, 4 and 6 h. No significant difference ($p>0.05$) at 0,
408 0.25, 1, 5, 8 and 24 h)

409 **Fig. 3 - Saliva nitrate (NO_3^-) level after administration of amaranth extract and placebo**
410 (highly significant difference ($p<0.001$) between amaranth and placebo group at all the time
411 points except 0 h and 24 h)

412 **Fig. 4 - Saliva nitrite (NO_2^-) level after administration of amaranth extract and placebo**
413 (highly significant difference ($p<0.001$) between amaranth and placebo group at all the time
414 points except 0 h and 24 h)

Table 1 - Pharmacokinetic parameters of nitrate and nitrite in plasma (n=16)

Parameters	Plasma nitrate		Plasma nitrite	
	Amaranth extract	**Placebo**	**Amaranth extract**	**Placebo**
AUC_{0-t} (μmol.h/ml) (Mean ± SEM)	3095.64 ± 179.58	1541.02 ± 102.76	7.87 ± 0.39	7.25 ± 0.36
C_{max} (μmol/L) (Mean ± SEM)	252.56 ± 8.60	69.34 ± 6.49	0.56 ± 0.06	0.36 ± 0.04
T_{max} (h)	1.00	1.50	0.50	0.50

Table 2 - Pharmacokinetic parameters of nitrate and nitrite in saliva (n=16)

Parameters	Saliva nitrate		Saliva nitrite	
	Amaranth extract	**Placebo**	**Amaranth extract**	**Placebo**
AUC_{0-t} (µmol.h/ml) (Mean ± SEM)	24017.47 ± 946.50	9129.54 ± 492.50	12035.16 ± 620.10	4992.94 ± 297.06
C_{max} (µmol/L) (Mean ± SEM)	3126.68 ± 331.11	519.77 ± 51.58	1080.51 ± 98.89	238.74 ± 9.39
T_{max} (h)	2.50	2.00	0.75	0.25

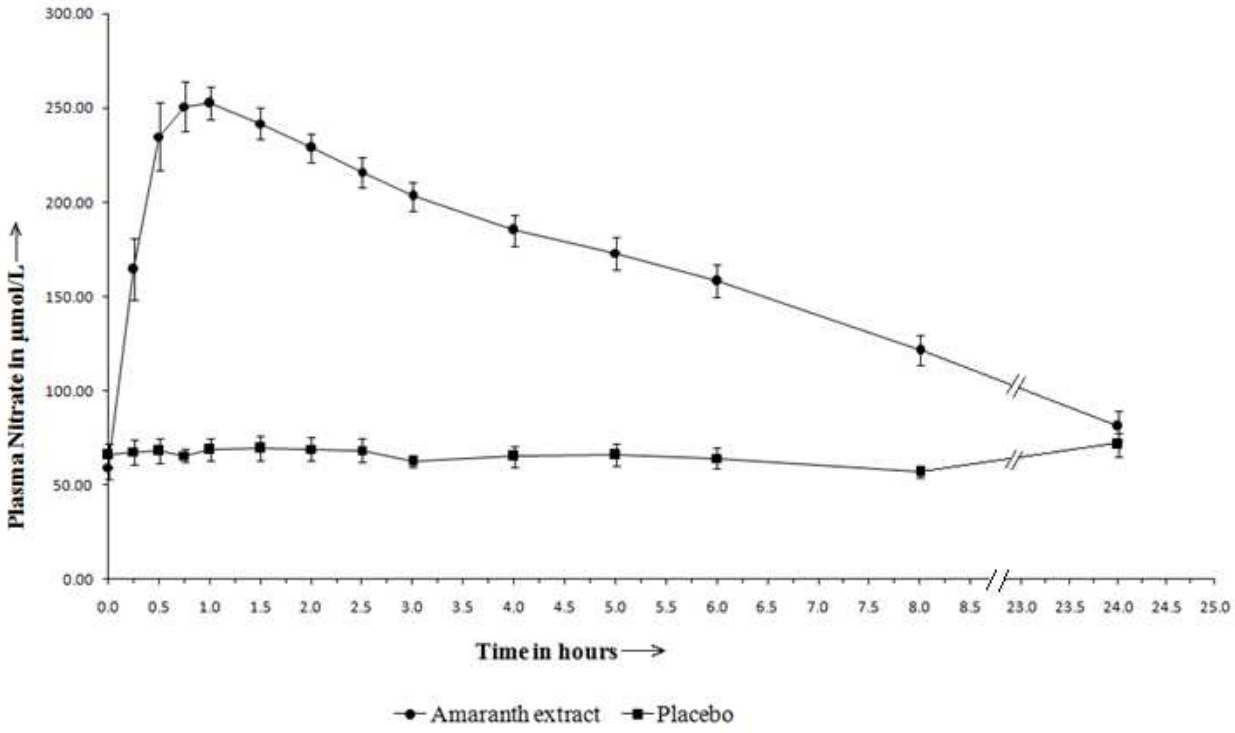

Fig. 1 - Plasma nitrate (NO_3^-) level after administration of amaranth extract and placebo (highly significant difference ($p<0.001$) between amaranth and placebo group at all the time points except 0 h and 24 h)

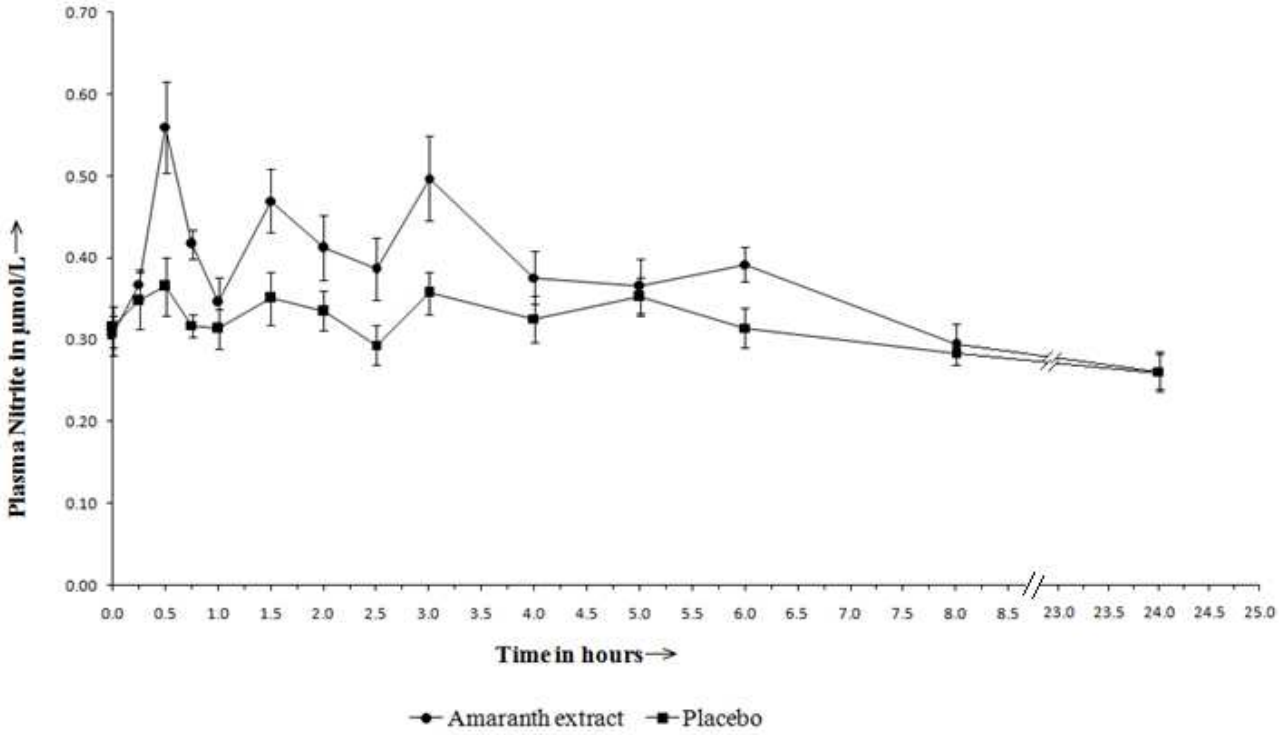

Fig. 2 - Plasma nitrite (NO_2^-) level after administration of amaranth extract and placebo (highly significant difference ($p<0.001$) between amaranth and placebo group at 0.5, 1.5 and 3 h. Significant difference ($p<0.01$) at 0.75, 2, 2.5, 4 and 6 h. No significant difference ($p>0.05$) at 0, 0.25, 1, 5, 8 and 24 h)

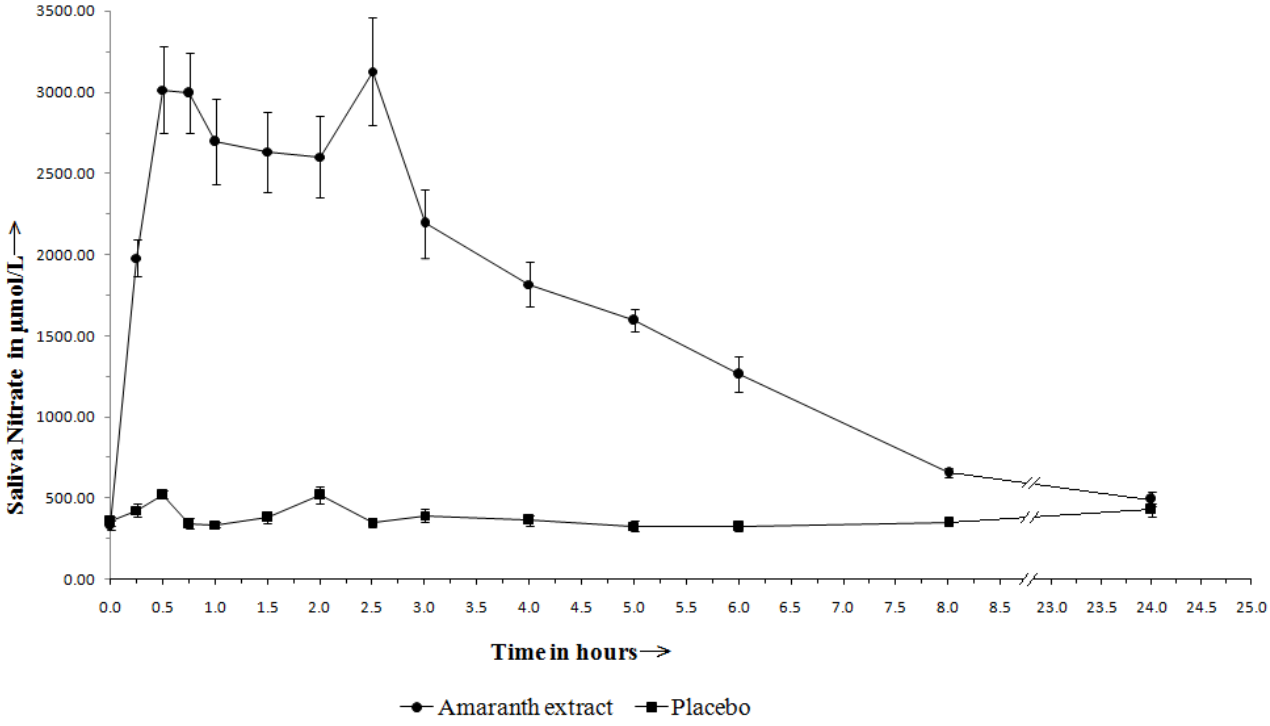

Fig. 3 - Saliva nitrate (NO_3^-) level after administration of amaranth extract and placebo (highly significant difference ($p<0.001$) between amaranth and placebo group at all the time points except 0 h and 24 h)

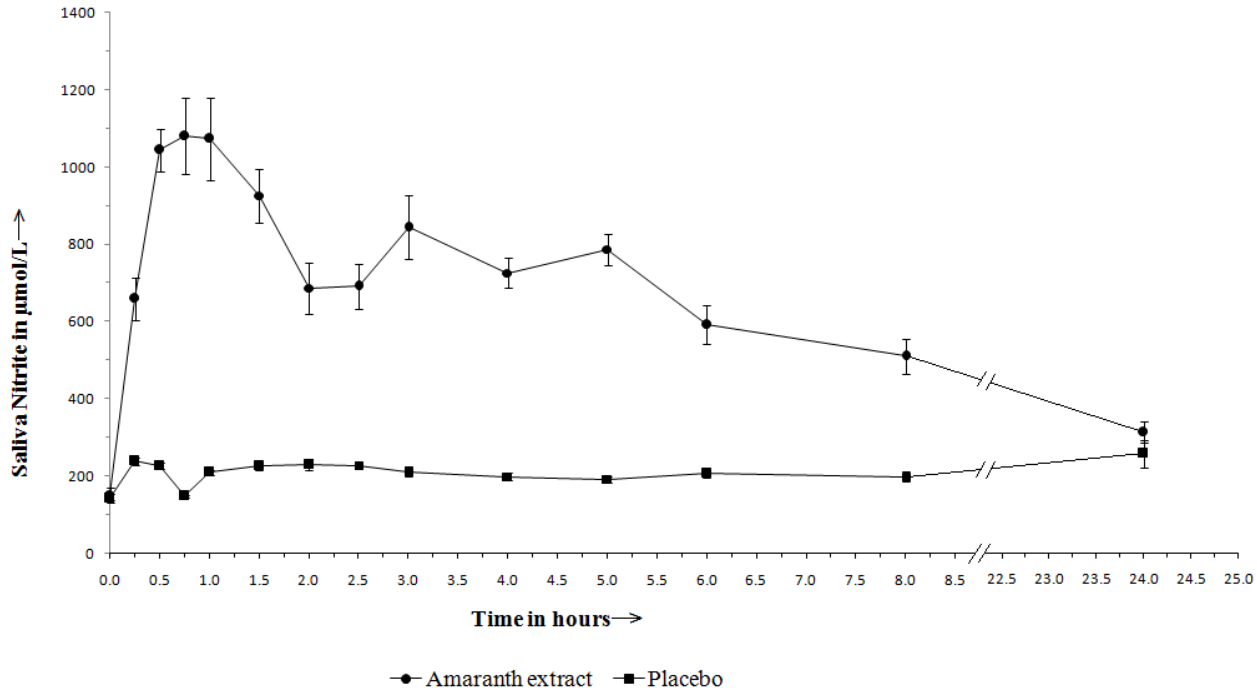

Fig. 4 - Saliva nitrite (NO_2^-) level after administration of amaranth extract and placebo (highly significant difference ($p<0.001$) between amaranth and placebo group at all the time points except 0 h and 24 h)

Highlights

- A human study on absorption of nitrate from Amaranth extract.
- Nitrate is converted into nitrite and then into nitric oxide.
- Amaranth extract increased the concentration of nitrate and nitrite in blood and saliva.