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Pharmacokinetic study of amaranth extract in healthy humans: A randomized trial

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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 aim of this 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 individuals 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^- levels in plasma as well as saliva were found to be significantly ($P < 0.001$) higher than in the placebo group. The NO_2^- level in plasma was slightly higher ($P < 0.05$) in the amaranth group (test group) compared with that in the placebo group, whereas the saliva NO_2^- level was significantly high ($P < 0.001$) in the amaranth extract–treated group than the 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 8 h. The increase in NO_3^- and NO_2^- levels can help to improve the overall performance of people involved in vigorous physical activities or sports.

Introduction

A diet rich in vegetables has been described as 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 $NO_3^- \rightarrow NO_2^- \rightarrow NO$ 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 on 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. A recent placebo-controlled study reported that beetroot juice supplementation significantly reduced the oxygen cost of treadmill walking and improved exercise tolerance in healthy young adults [14]. These results are remarkable because the oxygen uptake

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and work rate relationship have traditionally been considered to be independent of age, health status, and aerobic fitness [15]. The reduction in the oxygen cost of moderate-intensity exercise after dietary NO_3^- supplementation may be a result of a reduced adenosine triphosphate (ATP) cost of muscle force production [8], enhanced mitochondrial efficiency [16], or both. Dietary supplementation of NO_2^- and NO_3^- in mice has been shown to reverse endothelial dysfunction, suppress microvascular inflammation, and reduce levels 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]. Additionally, 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 to 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 sources of dietary NO_3^-. Amaranth (red spinach) is one such plant 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 southeast Asia and Latin America. The leaves and grains of amaranth are edible and contain large amounts of NO_3^- as well as other nutrients [23]. Amaranth leaves also are 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 or to result in clinical benefits. In a recent clinical study with 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 an NO_3^--rich dietary supplement (amaranth extract) is able to increase NO_3^- and NO_2^- levels in blood plasma and the saliva of healthy adults. The study was designed as a placebo-controlled, randomized, crossover study with 16 healthy adults.

Methods and materials

Medicament

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

Participants

We screened 23 individuals. Of these, 16 healthy adult males (age 18–40 y) met the inclusion criteria and were selected for the study. Study protocol was explained to all the participants and they willingly signed a consent form to participate in the trial. The study was approved by the ethics committee of Good Society for Ethical Research, Delhi (GSER/ND-2014/AP/03) and registered with Clinical Trials Registry-India.

Individuals between the ages of 18 and 40 y (both inclusive), weighing ≥50 kg, with body mass index (BMI) in the range of 18.5 to 30 kg/m^2, and who were able to provide written informed consent were included. The participants were of normal health as determined by medical history and physical examination, echocardiogram, chest x-ray (posteroanterior view), and laboratory tests that were performed 21 d before commencement of the study.

Individuals were excluded if they were incapable of understanding the informed consent process or not ready to sign informed consent; using organic nitrates; had significant history of hypersensitivity to leafy vegetable extract or amaranth; had signs or history of significant gastrointestinal, liver, or kidney disease; had significantly low or high blood pressure or any conditions known to interfere (e.g., taking any medicines or food supplements) with the absorption, distribution, metabolism, or excretion of amaranth extract. Individuals who had difficulty donating blood and those 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 arms and then crossed over after a 2 wk washout period. This ensured that all participants received each of the two interventions.

Participants checked in to the clinical facility at least 12 to 14 h before the test sample administration. They were not allowed to eat anything for 10 h before undergoing the baseline venous blood test. 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 participant at room temperature in sitting posture, in each period.

Postdose blood samples (6 mL each time) were collected at 15, 30, and 45 minutes and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 24 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 each time) also were collected in cryovials at the same time and stored at −80°C until analysis.

Food was restricted up to 6 h postdosing with the test sample. The limitation of drinking water was maintained for 2 h (1 h before dosing and 1 h 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 postdose, respectively, in each period of the study. Cleaning teeth, tongue, and 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 ultra-performance liquid chromatography (UPLC) method. 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 × 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 to 0.2 mL/min. Three mobile phases were used for elution. Mobile phase A was prepared by dissolving 1.4 g tetrabutyl ammonium hydroxide in high performance liquid chromatography (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 a 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 to 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 0.2 micron filter and used in the UPLC for direct injection to analyze NO_3^- at 222 nm. For the quantification of NO_2^-, a part of the supernatant liquid was derivatized with Griess reagent, injected into the UPLC and the chromatogram was monitored at 520 nm. Griess reagent comprises of sulfanilic acid (Griess A) and 1-naphthylamine (Griess B). This reagent converts NO_2^- into a purple azo compound, which is detectable by PDA detector and concentration of NO_2^- can be determined.

Pharmacokinetic and statistical analysis

The pharmacokinetic analysis was performed using noncompartment model by WinNonlin version 5.3 and parameters like C_{max}, T_{max} and area under the curve (AUC) were calculated. The data was analyzed for significance by one-way analysis of variance.

Results

Sixteen individuals were recruited for the study. All completed the period 1 study, whereas one dropped out in the second period of study for unknown reasons. Ingestion of amaranth extract/glucose powder was tolerated well by all participants. None of the participants reported any discomfort or side effects.

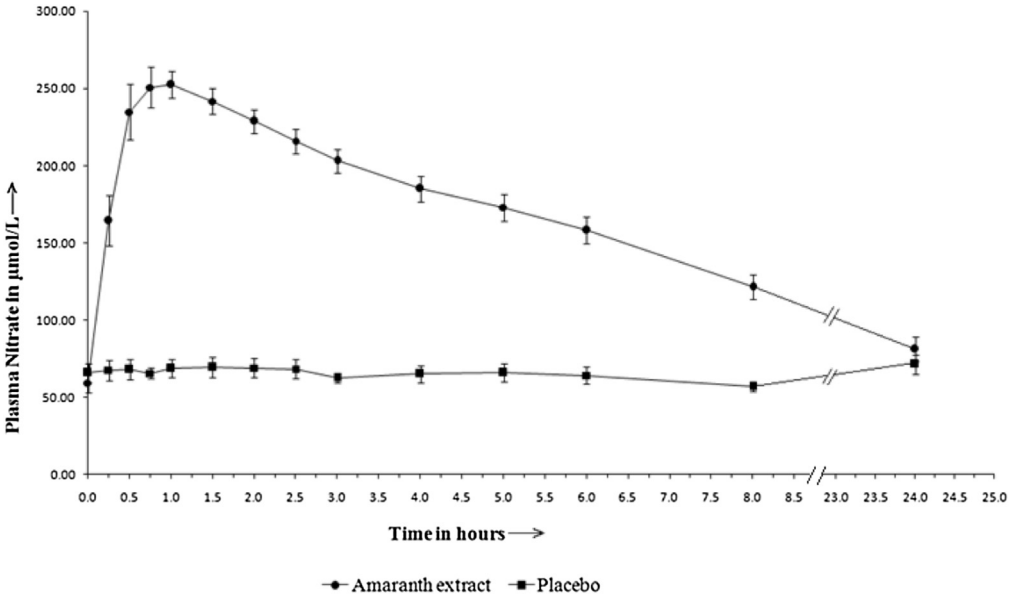

Fig. 1. Plasma nitrate (NO_3^-) levels after administration of amaranth extract and placebo (highly significant difference [$P < 0.001$] between amaranth and placebo groups at all time points except 0 and 24 h).

Plasma nitrate and nitrite

The mean plasma NO_3^- level after administration of amaranth extract and placebo are presented in Figure 1. There was no significant difference between treatments in the baseline (i.e., 0 h) plasma NO_3^- concentrations. After administration of amaranth extract, NO_3^- level increased significantly and the maximum concentration (252.56 ± 8.60 µmol/L) was observed at 1 h. Moreover, the level of NO_3^- in plasma remained significantly elevated ($P < 0.001$) for at least 8 h postdose. 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 h. The placebo was not able to increase the mean NO_2^- level in plasma significantly ($P > 0.05$) compared with baseline value.

Saliva nitrate and nitrite

Because about 30% of absorbed NO_3^- secretes into the saliva where it reduces into NO_2^- by oral facultative bacteria, saliva was analyzed for the presence of NO_3^- and NO_2^-. The mean level of NO_3^- in saliva after administration of amaranth extract and

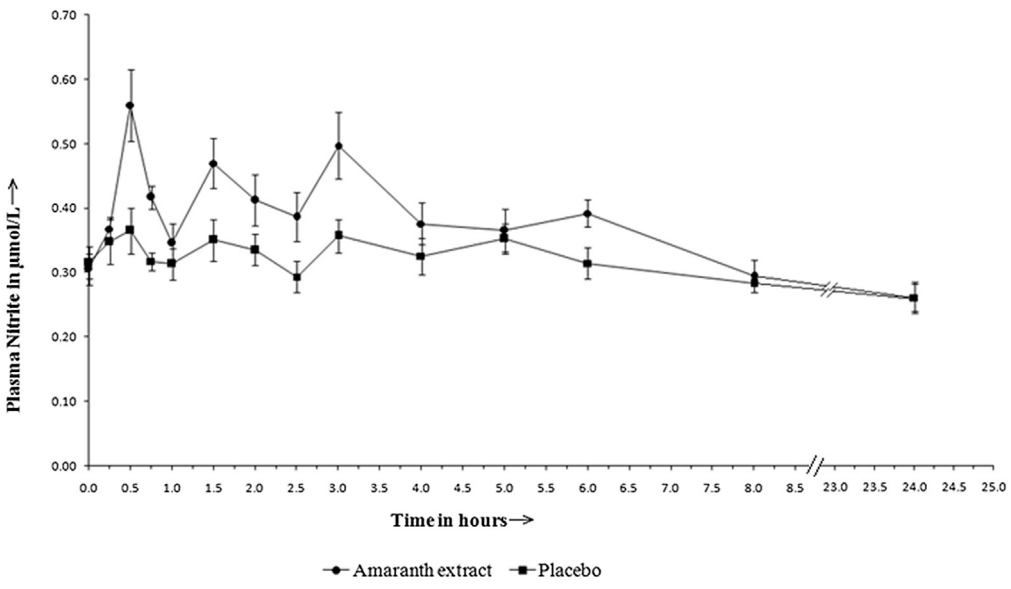

Fig. 2. Plasma nitrite (NO_2^-) levels after administration of amaranth extract and placebo (highly significant difference [$P < 0.001$] between amaranth and placebo groups at 0.5, 1.5, and 3 h; significant difference [$P < 0.01$] at 0.75, 2, 2.5, 4, and 6 h; no significant difference [$P > 0.05$] at 0, 0.25, 1, 5, 8, and 24 h).

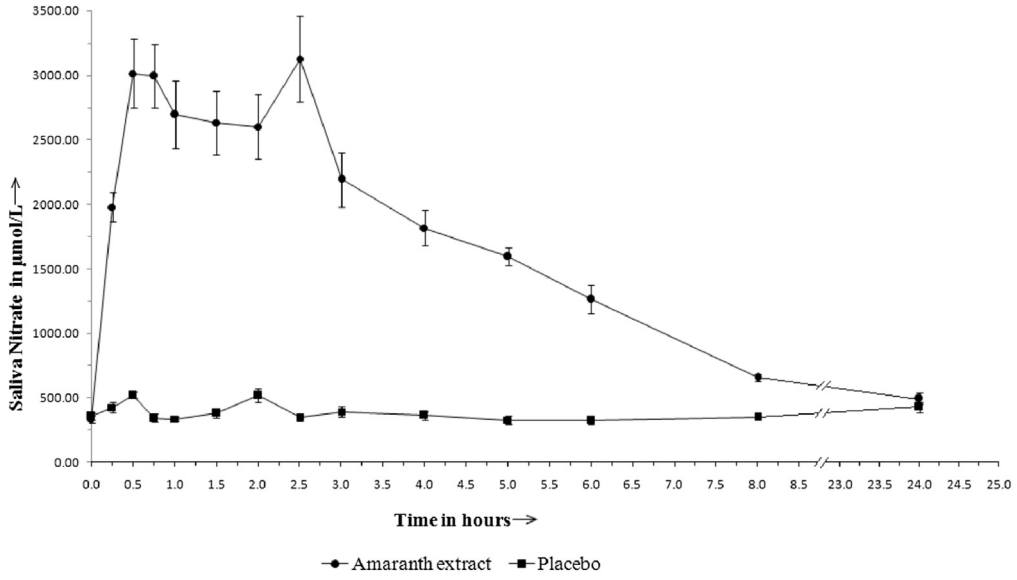

Fig. 3. Saliva nitrate (NO_3^-) levels after administration of amaranth extract and placebo (highly significant difference [$P < 0.001$] between amaranth and placebo groups at all time points except 0 and 24 h).

placebo are presented in Figure 3. 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 postdose. In the case of placebo, the mean NO_3^- level in saliva did not increase and remained almost the same as it was at baseline.

After the administration of amaranth extract, there was a significant increase in the concentration level of NO_2^- in the saliva ($P < 0.001$) compared with baseline values (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 ($P > 0.05$) compared with baseline.

Pharmacokinetic parameters

Pharmacokinetic parameters for NO_3^- and NO_2^- in plasma for the amaranth extract and placebo groups are presented in Table 1. $AUC_{0\text{-}t}$ for plasma NO_3^- in the amaranth extract and placebo groups was 3095.64 ± 179.58 and 1541.02 ± 102.76 $\mu mol \cdot h \cdot mL^{-1}$, respectively, which is highly significant ($P < 0.001$). C_{max} was 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

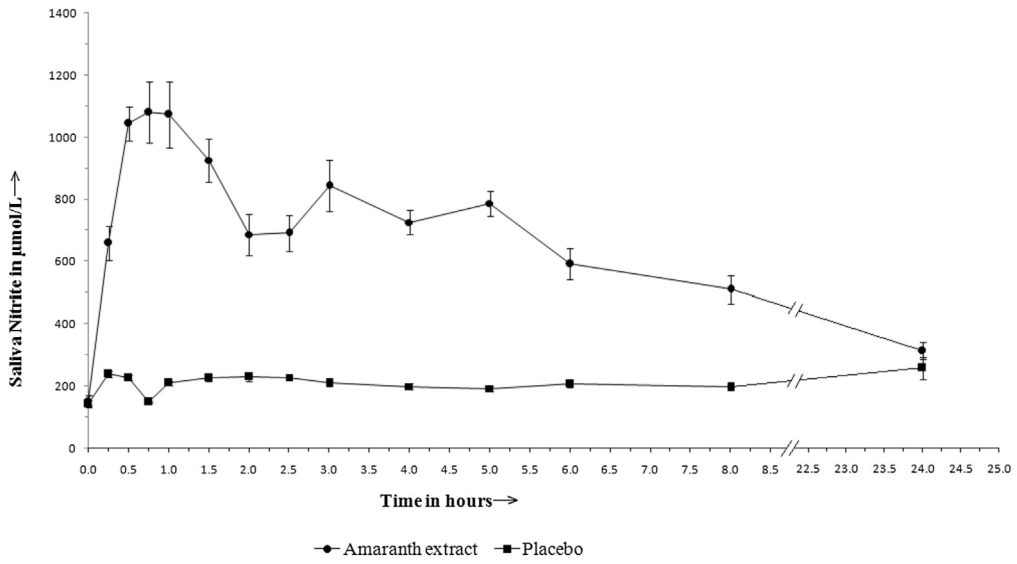

Fig. 4. Saliva nitrite (NO_2^-) levels after administration of amaranth extract and placebo (highly significant difference [$P < 0.001$] between amaranth and placebo groups at all time points except 0 and 24 h).

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

Parameters	Plasma nitrate		Plasma nitrite	
	Amaranth extract	Placebo	Amaranth extract	Placebo
$AUC_{0\text{-}t}$ (μmol·h·mL^{-1}) (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

that of the placebo group (0.36 ± 0.04 μmol/L). $AUC_{0\text{-}t}$ 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. $AUC_{0\text{-}t}$ of NO_3^- of the test group and the placebo group were 24017.47 ± 946.50 and 9129.54 ± 492.50 μmol·h·mL^{-1}, showing highly significant differences ($P < 0.001$) between the groups. In the same way, the 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 $AUC_{0\text{-}t}$ (12035.16 ± 620.10 and 4992.94 ± 297.06 μmol·h·mL^{-1} 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 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 cardiovascular 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 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; therefore, this strategy has failed in clinical trials [29].

Apart from patients suffering from endothelial dysfunction, athletes who exercise and perform physical work excessively, require more NO especially during hypoxia. In this study, the amaranth extract was 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. Because 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 ($P < 0.001$) compared with the placebo group. The concentration of NO_2^- in saliva reached the maximum in less than 1 h, 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]. Because 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, fluctuations 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 in NO_2^- levels in plasma.

In this study, none of the participants reported adverse events or any discomfort. The present study also confirmed the tolerability and safety of amaranth extract at the tested dosage (2 g) in humans. The pre- and post-study clinical parameters were not significantly different between participants.

A recent study on mice reported that dietary inorganic NO_3^- reverses features of metabolic syndrome in endothelial NOS-deficient mice [31]. This proof of concept has now been extended to individuals supplemented with dietary sources of NO_3^-. Dietary NO_3^- has 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,

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

Parameters	Saliva nitrate		Saliva nitrite	
	Amaranth extract	Placebo	Amaranth extract	Placebo
$AUC_{0\text{-}t}$ (μmol·h·mL^{-1}) (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

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 athletes or others partaking in 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 cardiovascular diseases in case of endothelial dysfunctions. It can be equally useful for athletes 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 8 h. 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. Because NO deficiency is one of the reasons for endothelial dysfunction and disorders related to aging, amaranth extract may be beneficial for the elderly.

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