

# Pharmacokinetics and Bioavailability of Annatto $\delta$ -tocotrienol in Healthy Fed Subjects

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## Abstract

**Background:** Although,  $\alpha$ -tocopherol is the most bioavailable form of vitamin E, but several animal and clinical studies have demonstrated tocotrienol bioavailability to various tissues. There are few reports on bioavailability of tocotrienols in humans. Most studies were carried out with mixtures of tocotrienols + tocopherols rather than pure tocotrienols. Moreover, dietary  $\alpha$ -tocopherol interferes with the bioavailability of tocotrienols, and prevents absorption and delivery to organs and tissues.

**Aim:** Pharmacokinetics and bioavailability of annatto-based  $\delta$ -tocotrienol, plasma levels of  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienol and tocopherols were quantified. In addition, several cytokines and microRNAs were examined.

**Study design:** An open-label, randomized study evaluated pharmacokinetics and bioavailability of  $\delta$ -tocotrienol in 33 healthy fed subjects. All subjects (11/dose) were randomly assigned to doses of 125, 250, or 500 mg/d. Plasma samples collected at 0, 1, 2, 3, 4, 6, 8, 10 h intervals were estimated by HPLC for tocols (tocotrienols and tocopherols).

**Results:** The present study describes the effects of  $\delta$ -tocotrienol on pharmacokinetic parameters of all eight tocol isomers. Supplementation of 125, 250 and 500 mg/d doses resulted in dose- dependent increases of (a) area under concentration-time curve ( $AUC_{0-110}$ , ng/ml) 2464, 5412, 14986; (b) maximum concentration ( $C_{max}$ , ng/ml) 829, 1920, 3278 ( $P < 0.001$ ); (c) time to achieve maximum peak ( $T_{max}$ , h) 3, 3, 6; (d) elimination of half-life ( $t_{1/2}$ , h) 1.74, 1.39, 2.54; (e) time of clearance (Cl-T,  $h^{-1}$ ) 0.049, 0.045, 0.030; (f) volume of distribution (Vd/f, mg/h) 0.119, 0.114, 0.113; and (g) elimination rate constant ( $K_e$ ,  $h^{-1}$ ) 0.412, 0.401, 0.265. Similar results were reported for the other tocols. Maximum plasma levels of  $\delta$ -tocotrienol were observed at 3 h with doses of 125 and 250 mg/d, and 6 h with 500 mg/d.  $\gamma$ -tocotrienol,  $\beta$ -tocotrienol,  $\alpha$ -tocotrienol,  $\delta$ -tocopherol,  $\gamma$ -tocopherol  $\beta$ -tocopherol and  $\alpha$ -tocopherol were appeared in the plasma after 2 h. Moreover,  $\delta$ -tocotrienol treatment resulted in down-regulation of eight cytokines and upregulation of adiponectin, TGF- $\beta$ 1, and leptin. The expression of miR-34a (increased in bipolar disorder) was down-regulated, but expression of miR-107, miR-122a, and miR-132 (decreased in Alzheimer's disease) was up-regulated by  $\delta$ -tocotrienol treatment.

**Conclusion:** This is the first study describing the effect of  $\delta$ -tocotrienol on pharmacokinetics and bioavailability of all eight tocol isomers. When tocotrienols are supplemented in absence of tocopherols,  $\delta$ -tocotrienol has better bioavailability, and  $\delta$ -tocotrienol is converted stepwise to other tocotrienols/tocopherols. These results support that tocotrienol, particularly  $\delta$ -tocotrienol, as a dietary supplement might be useful in the prevention of age-related and chronic ailments.

**Keywords:** Annatto-based  $\delta$ -tocotrienol; Bioavailability; AUC;  $C_{max}$ ;  $T_{max}$ ; Vd/L; Cl/h;  $t_{1/2}$ ;  $K_e$ ; Tocopherols; Tocotrienols; microRNAs

## Abbreviations

AUC=Area under Concentration-Time Curve;  $C_{max}$ =Concentration Maximum;  $T_{max}$ =Time to reach Maximum Concentration; Vd/L=Apparent Volume of Distribution; Cl-T (Cl/h)=Time of Clearance;  $t_{1/2}$  (t/h) = Half-life time;  $K_e$  ( $h^{-1}$ )=Elimination Rate Constant.

## Introduction

Several studies have reported the antioxidant, anti-inflammatory, anticancer, hypocholesterolemic and neuroprotective properties of tocotrienols in different cell lines, animal models, and in humans [1-8]. However, question on the bioavailability of pure tocotrienols remained unanswered. Therefore, it is important to understand the absorption and bioavailability mechanism of tocotrienols before carrying out investigations into the therapeutic efficacy in humans. The bioavailability of naturally-occurring tocotrienols differ considerably in their absorption, therefore therapeutic uses of tocotrienols remain

controversial. It was reported that after feeding rats mixed tocotrienols, the oral bioavailability of  $\alpha$ -tocotrienol was 28% compared to 9% of  $\gamma$ -tocotrienol and  $\delta$ -tocotrienol [9]. Tocotrienols in humans were detected in postprandial plasma [10,11], and they were found enriched in triacylglycerol-rich particles, HDL, and LDL after administration of palm tocotrienol-rich fraction (mixture of 68% tocotrienol + 32%

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$\alpha$ -tocopherol). The key parameter of bioavailability determination, the total area under the concentration-time curve ( $AUC_{0-\infty}$ , h) for plasma  $\alpha$ -tocotrienol, was 60% larger than for  $\gamma$ -tocotrienol [11].

It was also reported that the bio-discrimination of  $\alpha$ -tocopherol (vitamin E) influences the rate of tocotrienol absorption, due to high affinity of  $\alpha$ -tocopherol with "alpha-tocopherol transfer protein" ( $\alpha$ -TTP), which mediates secretion of  $\alpha$ -tocopherol (100%) from the liver into the circulatory system, and is much higher than  $\alpha$ -tocotrienol (12%) or other tocotrienols [12]. Moreover,  $\alpha$ -tocopherol has been reported to attenuate the cholesterol-lowering effect of tocotrienols through activation of the HMG-CoA reductase activity (whereas tocotrienols have a desirable inhibiting effect on its activity) [12,13]. Also  $\alpha$ -tocopherol interferes with tocotrienol functions such as attenuation of cancer inhibition [14-16], exacerbation of stroke injury [17], inhibition of absorption [18], and induction of tocotrienol catabolism [19]. Therefore, dietary supplementation of tocotrienol preparations with minimal concentrations of tocopherol (<20%) in the mixture has been recommended for the inhibition of several biological activities [12,20]. All the isomers of tocotrienols were detected in plasma as well as in various tissues of rats (liver, heart, and adipose) [21] and humans (skin, adipose, brain, cardiac muscle, and liver) [22]. Moreover, high doses of tocotrienols may be useful for cancer chemoprevention and treatment. Large doses of  $\delta$ -tocotrienol up to 3200 mg/d have been used to treat patients suffering from resectable pancreatic cancer [23].

The biological properties of tocotrienols have been underestimated despite several studies showing their positive effects on physiological and biological functions [1-8]. The scientific recognition for tocotrienols remains limited as compared to tocopherols due to their low concentrations in most food products. Although they are present in high concentrations in palm oil and annatto seeds, the latter being the richest source of  $\delta$ -tocotrienol without any tocopherols. In addition, there is a lack of information on the bioavailability and metabolism of tocotrienols in humans. The absorption of tocotrienols was negligible via intraperitoneal and intramuscular route, while incomplete absorption was observed when given via the oral route in rats [9]. Tocotrienols were also administered intravenously (tail vein injection) by encapsulation into transferrin-bearing vesicles [24]. Moreover, when tested in a tumor-targeted vesicle system, tocotrienols are very promising as a potential therapeutic system to eradicate human epithelial tumors and melanoma tumors in murine xenografts [25]. There is a limited amount of literature describing the bioavailability of tocotrienol, particularly

in humans [11,20] and most of the investigations focused on the bioavailability of palm tocotrienol-rich fraction, which is a tocotrienol-tocopherol mixture. One study explored the bioavailability of barley and palm oil tocotrienols from which tocopherol had been removed, and compared them to bioavailability of tocopherol-tocotrienol mixtures with the observation that alpha-tocopherol may negatively impact tocotrienol absorption [26].

Tocotrienols from annatto containing 10%  $\gamma$ -tocotrienol + 90%  $\delta$ -tocotrienol (Figure 1) without tocopherols, were not previously tested for their bioavailability and pharmacokinetics. The pharmacokinetics results of tocotrienols (mixture of tocopherols plus tocotrienols) in healthy humans were obtained under fed condition as compared to fasting state [27]. Tocopherols have been shown to prevent absorption and organ/tissue delivery of tocotrienols [18,28]. In addition, evidence suggests that when administered at high doses, tocotrienols may convert to  $\alpha$ -tocopherol [29]. The present study was carried out to determine the pharmacokinetics and bioavailability of various doses of annatto-based  $\delta$ -tocotrienol in healthy volunteers under fed condition, and also to determine the plasma levels of  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienols and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherols. Additionally, the present study examined the effects of  $\delta$ -tocotrienol on several cytokines/proteins, and circulating microRNAs, which are small non-coding RNAs involved in many biological processes.

## Materials and Methods

### Reagents

DeltaGold 125 mg soft gels from annatto seeds (composition 90%  $\delta$ -tocotrienol + 10%  $\gamma$ -tocotrienol) were supplied by American River Nutrition, Inc. (Hadley, MA, USA). HPLC grade- hexane, isopropyl alcohol, methyl alcohol, water, ascorbic acid, and butylated hydroxyl toluene were purchased from Sigma Chemical Co. (St. Louis, USA).

### Supplies for HPLC

Fisher brand disposable borosilicate glass tubes Screw cap, 20  $\times$  150 mm; Borosilicate screw cap with conical shape-10 ml; Screw caps with Teflon insert; Disposable glass pipettes 1 ml, Rubber teats; Plastic test tube holders for above glass tubes; Test tube Shaker; Buchner test tubes Vacuum Evaporator (48 centrifuge test tube 10 ml) holder; Water vacuum Aspirator; Solvent dispensing bottles units (1000 ml).

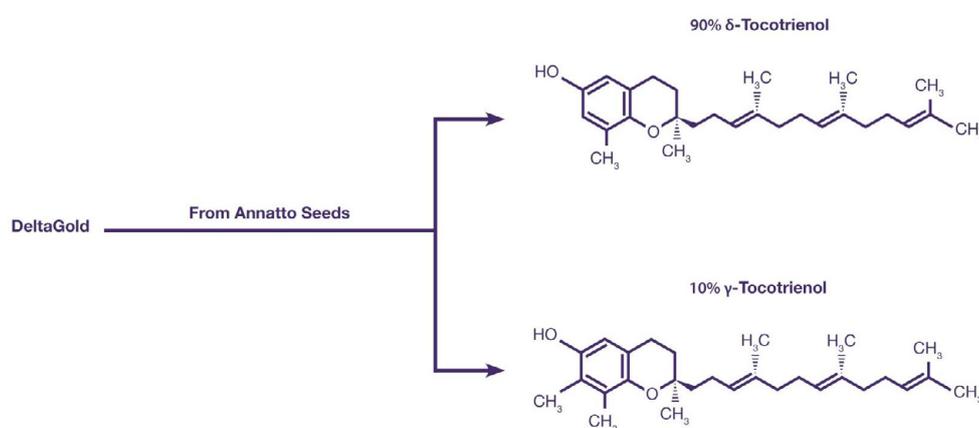


Figure 1: Chemical structures of DeltaGold components ( $\delta$ -tocotrienol-90% +  $\gamma$ -tocotrienol-10%). Similar Figure has been published, Qureshi AA *et al.* *British Journal of Medical Research*, 6(4): 351-366, 2015.

## Study subjects

The present investigation was a single-center, open-label, randomized study to determine the systemic pharmacokinetics and bioavailability of annatto-based  $\delta$ -tocotrienol after oral supplementation to 33 healthy subjects under fed condition, using doses of 125 mg, 250 mg, and 500 mg (11 subjects/dose). The study was conducted in accordance with the current Good Clinical Practices (FDA, 1996) and the Declaration of Helsinki (WMA, 2008). The study protocol was approved by the institutional review board (IRB) of the Pakistan Ordinance Factory (POF) Hospital, Wah Cantt, Pakistan. The study was carried out under FDA approved "Investigational New Drug" (IND) number 36906.

Thirty three adult healthy male subjects were recruited for the study according to the guidelines provided by the United States Food and Drug Administration (FDA, 2003) from Wah Cantt, Pakistan. All participants of study signed an informed consent. All subjects were male between 18-50 years and weighed within 20% of normal body weight according to the Metropolitan Life Assurance Tables. Clinical history and physical examination of all participants was carried out by a consultant physician in the hospital. Systolic and diastolic blood pressures were measured at rest position. All relevant investigations, including fasting plasma glucose, complete liver function tests (LFTs), and serum urea, were analyzed for screening of participants. The human subjects who had acute or chronic disease, malabsorption, cholecystectomy, or were currently taking vitamin E supplements were excluded from the study.

## Study design

An open-label, randomized study was carried out to determine the pharmacokinetics and bioavailability of annatto-based  $\delta$ -tocotrienol after oral supplementation in 33 healthy male subjects under fed condition. All the subjects were fed Pakistani heavy breakfast comprising of fruit cocktail, halwa, puri, paratha, omelet, orange juice and tea. The volunteers were randomly assigned to one of the tocotrienol soft gel dose levels (125 mg, 250 mg, and 500 mg), which they received once after breakfast. Restricted foods included nuts, cereals and vegetable oils. The food was prepared in "Army Mess" under strictly hygienic conditions. These conditions are known to be optimal in securing the best results. In addition to breakfast, participants received lunch that included chicken biryani, mutton (lamb) qurma, sheermal/nan, and gajar ka halwa (desert), and dinner that included fried Basmati rice, kabab (chicken), rogni nan, shahi tukray, and kasmiri tea with pistachios. Fruit cocktail was served with every meal.

## Blood Sample Collection and Extraction

On the study day each subject consumed one dose of  $\delta$ -tocotrienol immediately after consuming a typical heavy Pakistani breakfast. During the experimental period (0 h - 10 h), subjects consumed a good lunch, evening tea, and dinner as described above. All subjects were allowed to drink water freely. For the determination of the pharmacokinetic profile of tocotrienols in plasma, venous blood samples (2  $\times$  5 ml) were collected in EDTA glazed tubes at pre-dose (0 h) and at post-dose (1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 10 h). The samples were then centrifuged at 3000  $\times$  g for ten minutes. Processed plasma samples were stored in Eppendroff tubes at -80°C till further analysis.

## High Performance Liquid Chromatography Analysis

Highest purity HPLC grade solvents and reagents were used. The reagents consisted of absolute ethanol and methanol (Fisher Scientific,

Pittsburgh, PA). Hexane, Ascorbic Acid and butylated hydroxyl toluene (BHT) were obtained from Sigma Chemical Co. Inc. (St. Louis, MO). The various tocopherol ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -) and tocotrienol ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -) standards were obtained from ChromaDex Inc. (10005 Muirland Blvd, Suite G, Irvine, CA). The working standards were prepared by mixing appropriate amounts of the stock solutions. The working standards used for repeated determinations were stored at -20°C for a maximum period of 24 h.

Following solutions were prepared for HPLC analysis of various tocols.

- 1% ascorbic in HPLC absolute ethanol
- 10 mg/10 ml butylated hydroxyl toluene in HPLC absolute ethanol
- Stock solutions of tocols. 1 mg/ml each standard tocol was prepared in HPLC hexane=A
- Working tocols solution: 10  $\mu$ g/ml of each tocol (10  $\mu$ l of A) plus 990  $\mu$ l hexane was prepared=B
- Working tocols solution: 1  $\mu$ g/ml of each tocol (100  $\mu$ l of B) plus 900  $\mu$ l hexane
- Working tocols mixture: 10  $\mu$ g/ml or 1  $\mu$ g/ml mixture of all eight tocols standards depending on the sensitivity of fluorescence of HPLC detector
- The solution should be kept at -20°C

The extraction of plasma was carried out for the estimation of  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherols and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienols (tocols) by modified normal and reverse phase HPLC procedures as described [30,31]. Briefly, plasma (200  $\mu$ l) was added in screw cap disposable glass tube (15 ml) + 200  $\mu$ l 1% ascorbic acid + 25  $\mu$ l butylated hydroxyl toluene (1 mg/1 ml) + 900  $\mu$ l HPLC water + 5 ml HPLC hexane, tube was closed and shaken for 10 minutes on a shaker, and centrifuged for 10 min at 5000 rpm. The upper layer was transferred to a centrifuge tube (10 ml) with a glass pipette, and hexane was removed under vacuum at 40°C using a water aspirator. Hexane (200  $\mu$ l) was added, shaken (vortex) for 30 s, centrifuged (2000 rpm/5 min), and the solution was transferred into HPLC injecting vial (0.3  $\mu$ l). A normal phase silica column (5 micron, 30 cm  $\times$  4.0 mm I.D. obtained from Waters Associates, Milford, MA, USA) attached to a Guard column was used to separate various tocols. The High Performance Liquid Chromatography (HPLC) system consisted of a continuous-flow 307 pump (Gilson, Madison, Wisconsin, USA). The mobile phase was pumped at a flow rate of 1 ml-1.3 ml/min depending upon the elution of tocols. The  $\alpha$ -tocopherol typically eluted within 5-6 min, and  $\delta$ -tocotrienol at 18 min-20 min, with pressure varying between 0.4-0.5 psi under these conditions. A Shimadzu fluorescence monitor Model RF-535 set at excitation wavelength of 296 nm and an emission wavelength of 330 nm was utilized, and the peak areas were determined by Shimadzu Integrator-Model C-R3A (Shimadzu, Wood Dale, IL, USA). The eluting solvent was 0.3%-0.5% (vol/vol) isopropyl alcohol in hexane. The extract (20  $\mu$ l) of the sample was introduced into the column through the 10  $\mu$ l loop of Gilson's autosampler-231 injector. All the samples were analyzed in duplicate or triplicate. The retention time of the individual peaks of the unknown tocols were compared against the retention time of the pure standard tocols. The tocols were eluted under these conditions in the sequence:  $\alpha$ -tocopherol----->  $\alpha$ -tocotrienol----->  $\beta$ -tocopherol----->  $\beta$ -tocotrienol----->  $\gamma$ -tocopherol----->  $\gamma$ -tocotrienol----->  $\delta$ -tocopherol----->  $\delta$ -tocotrienol [30].

#	Parameters	$\delta$ -tocotrienol (125 mg)	$\delta$ -tocotrienol (250 mg)	$\delta$ -tocotrienol (500 mg)
1	Age (Years)	33.45 $\pm$ 9.49'	36.27 $\pm$ 9.47'	34.64 $\pm$ 10.53'
2	Males (n)	11	11	11
3	Height (cm)	165.9 $\pm$ 0.36	169.4 $\pm$ 0.21	168.3 $\pm$ 0.16
4	Weight (kg)	77.09 $\pm$ 13.90	76.73 $\pm$ 12.52	74.18 $\pm$ 9.16
5	BMI (kg/m <sup>2</sup> )	28.32 $\pm$ 1.54	27.50 $\pm$ 1.33	26.30 $\pm$ 1.28
6	Pulse (Min)	78 $\pm$ 6	77 $\pm$ 5	79 $\pm$ 7
7	Systolic BP (mmHg)	126.43 $\pm$ 6.81	124.83 $\pm$ 8.17	121.82 $\pm$ 6.22
8	Diastolic BP (mmHg)	81.23 $\pm$ 5.41	79.80 $\pm$ 6.74	80.11 $\pm$ 5.90
9	Serum Creatinine ( $\mu$ L)	99.12 $\pm$ 10.30	97.34 $\pm$ 9.68	96.77 $\pm$ 8.64
10	Serum ALT (U/L)	22 $\pm$ 9	28 $\pm$ 7	23 $\pm$ 10
11	Serum Glucose (mmol/L)	4.10 $\pm$ 0.92	4.10 $\pm$ 0.94	4.10 $\pm$ 0.92
12	Serum Total Cholesterol (mmol/L)	4.61 $\pm$ 0.95	5.04 $\pm$ 0.98	4.92 $\pm$ 0.94
13	Serum Triglyceride (mmo/L)	1.53 $\pm$ 0.67	1.47 $\pm$ 0.71	1.59 $\pm$ 0.85

**Table 1:** Baseline characteristics of pharmacokinetic study subjects (n=11/treatment). Values represent Standard Deviation ( $\pm$  SD)

The separation of various tocots was also carried out by reverse phase Kinetex 2.6  $\mu$  PFP 100A column (150  $\times$  4.6 mm) attached to Security Guard ULTRA Cartridges (UHPLC PFP for 4.6 mm ID column). The eluting solvent was methanol + water (85% + 15%, vol/vol) at a flow rate of 0.8 ml/min [30]. The rest of the conditions were the same as described above. The tocots elution sequence was;  $\delta$ -tocotrienol---->  $\beta$ -tocotrienol---->  $\gamma$ -tocotrienol---->  $\alpha$ -tocotrienol---->  $\delta$ -tocopherol---->  $\beta$ -tocopherol---->  $\gamma$ -tocopherol---->  $\alpha$ -tocopherol (vitamin E; 30).

### Estimation of human plasma cytokines/proteins and miRNA

The various plasma cytokines/proteins were estimated by using Human Cytokine Elisa Plate Array I (chemiluminescence), Catalog number EA-4001 (Signosis, Inc., Santa Clara, CA, 95054). Assays for estimating the plasma cytokines/protein were carried out according to the protocols provided by Signosis, Inc. The incubation of each assay mixture at various temperatures was carried out by using Enviro-Genie Shaker/incubator (Enviro-Genie Industries, Bohemia, NY). The intensity of chemiluminescence was detected using a Microplate Luminometer (GloMax Promega, Madison, WI) at 500 nm, and luminescence was monitored over 20 min period. Estimation of circulating miRNAs was carried out using "Customized miRNA Direct Hybridization Plate Array", chemiluminescence; Catalog Number Inv-00465 (Signosis, Inc., 1700 Wyatt Drive Suite 10-12. Santa Clara, CA).

### Pharmacokinetic analysis

The computer software PKSolver 2.0 was used for calculation of pharmacokinetic parameters through standard two compartmental analysis of each subject in each group for area under the curve ( $AUC_{0-t_{10}}$ ,  $AUC_{0-\infty}$ ), maximum plasma concentration ( $C_{max}$ ), time to reach maximum concentration ( $T_{max}$ ), half-life time ( $t_{1/2}$ ; h), and time of clearance (Cl-T). The values for these parameters were based on mean concentrations of each time point of each isomer ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) of tocotrienols (T3) and tocopherols (T) and also using the values of each subject in each group. Data of plasma tocotrienol concentrations versus time was used to calculate pharmacokinetic parameters. AUC from 0 h-time to 10 h-time ( $AUC_{0-t_{10}}$  h) for plasma tocotrienols was calculated by trapezoidal rule, where t is the last measured time point. The  $C_{max}$  and  $T_{max}$  were obtained directly by inspecting each individual plasma level-time curve, and also by GraphPad Prism 5. The AUC values for each isomer were also checked by using following equation: ( $\alpha$ -T3/T baseline [0-time] +  $\delta$ -T3/T1 h)/2 + ( $\delta$ -T3/T 1 h +  $\delta$ -T3/T 2 h)/2 + ( $\delta$ -T3/T 2 h +  $\delta$ -T3/T 3 h)/2 + ( $\delta$ -T3/T 3 h +  $\delta$ -T3/T 4 h)/2 +

( $\delta$ -T3/T 4 h +  $\delta$ -T3/T 6 h)/2 + ( $\delta$ -T3/T 6 h +  $\delta$ - T3/T 8 h)/2 +( $\delta$ -T3/T 8 h +  $\delta$ -T3/T 10 h)/2'. The values of apparent volume of distribution (Vd/f; ml instead per liter) of each subject were calculated by using the equation: oral dose  $\times$  f (where f bioavailability is 1)/AUC  $\times$  Ke, and Ke values were calculated by equation: time of clearance (Cl-T)/Vd/f).

### Statistical Analysis

Data was analyzed using SPSS 22 version (SPSS Inc, Chicago). Descriptive statistics comprising mean and SD were calculated. Percent difference was calculated from baseline value of each analyte. Repeated two-way ANOVA was applied on dose response data. P value of <0.05 was considered significant. Normal distributed variables were summarized as means  $\pm$  SD, and percent differences were calculated from baseline values of each inflammatory marker by one-way variance. Paired Student's t-test was applied for normally distributed variables for percentage values of cytokines/proteins, and miRNAs. A two tailed P value <0.05 was considered significant. Data are reported as mean  $\pm$  SD (Standard Deviation).

### Results

The physical characteristics of all the participants were reported in Table 1. The ages, heights, weights, systolic, diastolic blood pressures, and other parameters of participants in each dose were closely similar to avoid variation in the results due to differences in the characteristics of each dose treatment (Table 1).

The pharmacokinetics and bioavailability of a compound required estimations of plasma total area under the concentration-time curve ( $AUC_{0-t_{10}}$  or  $\infty$ ), plasma maximum concentration ( $C_{max}$ ), time to reach maximum plasma concentration ( $T_{max}$ ), the apparent volume of distribution (Vd/l), time of clearance (Cl-T, Cl/h), half-life time ( $t_{1/2}$ ; t-h), and elimination rate constant (ke; h<sup>-1</sup>). Among them, general emphasis was focused on three parameters [( $AUC_{0-\infty}$ ), ( $C_{max}$ ), and ( $T_{max}$ )] to establish the pharmacokinetics and bioavailability of the compound.

Significant differences were observed between logarithmic converted values of  $AUC_{0-t_{10}}$ ,  $AUC_{0-\infty}$ ,  $C_{max}$ , and  $T_{max}$  for  $\delta$ -,  $\gamma$ -,  $\beta$ -,  $\alpha$ -tocotrienol and  $\delta$ -,  $\gamma$ -,  $\beta$ -,  $\alpha$ -tocopherol. The estimation of these parameters were based on the quantitative estimation of eight isomers of each subject's plasma tocots ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienol and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherol) separated by HPLC at 0 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 10 h time for dose of 125 mg (n=11), 250 mg (n=11), and 500 mg (n=11).

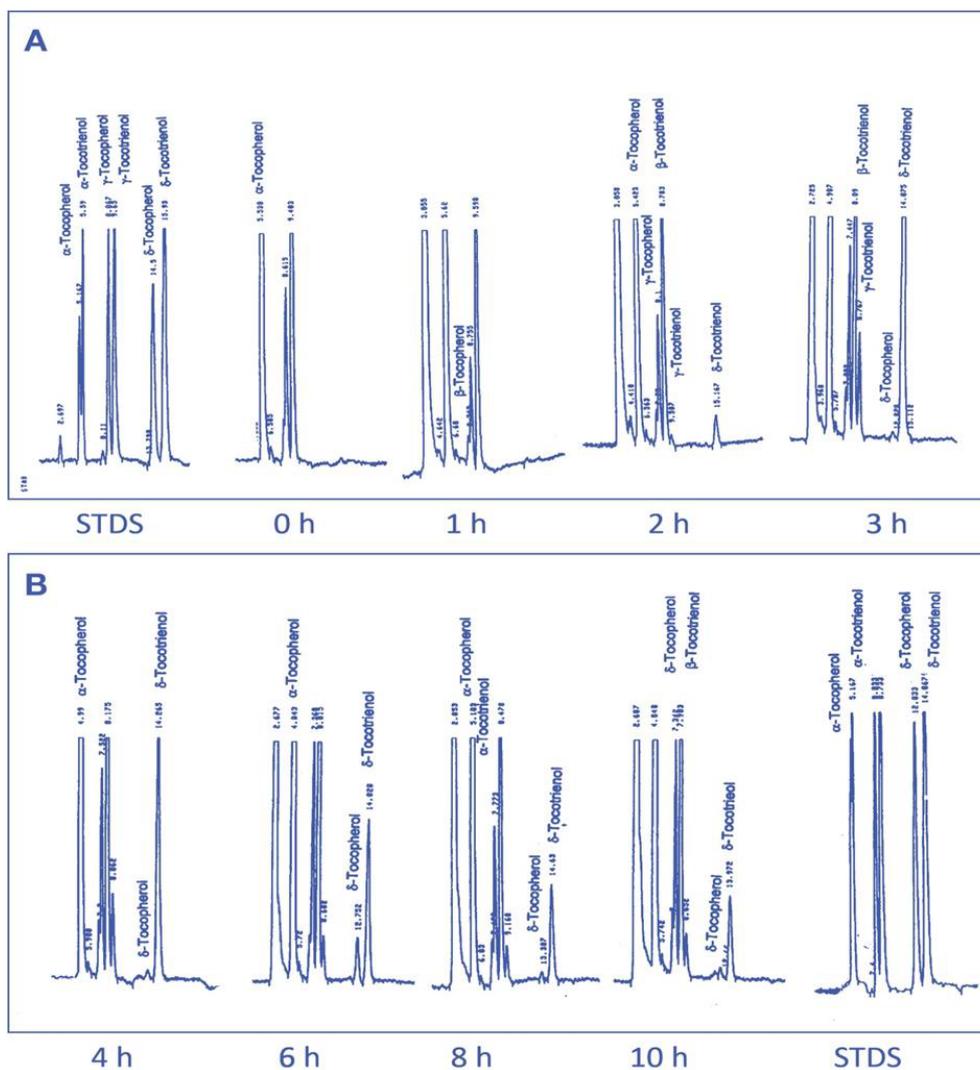
This is the first report describing the quantitative estimation of

all eight isomers of tocols (four tocopherols plus four tocotrienols) from human plasma samples after administering annatto-based  $\delta$ -tocotrienol at 125 mg, or 250 mg, or 500 mg doses at 0 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, and 10 h time periods. The HPLC was carried out by using normal phase silica and reverse phase C18 columns of plasma samples collected at these intervals (8 samples/subject) for the determination of pharmacokinetics of various tocols. The separation of all isomers of tocols was obtained as baseline of all the samples on a normal phase silica column under present conditions (Figure 2).

In HPLC analyses, at 0 h and 1 h time period, no peaks of  $\delta$ -tocotrienol and  $\delta$ -tocopherol were observed in plasma samples of 125 mg dose participants (Table 2). Similarly, plasma samples of 250 mg and 500 mg doses of  $\delta$ -tocotrienol and  $\delta$ -tocopherol at 0 h showed no peaks, but at 1 h time period, out of 11 samples of each group, only 3 samples of the 250 mg dose and 4 samples of the 500 mg dose showed small concentrations of  $\delta$ -tocotrienol,  $\alpha$ -tocotrienol and  $\delta$ -tocopherol (Table 2). For 0 h only  $\alpha$ -tocopherol,  $\beta$ -tocopherol,  $\gamma$ -tocopherol and

$\beta$ -tocotrienols were observed for the samples of all three doses (Tables 2A-2C). It is clear from Figure 2A that  $\delta$ -tocotrienol appeared after 2 h with all three doses, reached maximum at 3 h, and that  $\delta$ -tocopherol also appeared at 3 h. Both of these levels started declining between 3 h and 6 h (Table 2 and Figure 2B). Similar patterns of HPLC profile were noted throughout HPLC analyses of all the samples (Figures 2A and 2B). The results obtained with the reverse phase C18 column were comparable to those of the normal phase silica column. However, the elution profiles of all subjects showed better baseline separation with normal phase silica column [30] (data was not shown).

The plasma values and their corresponding standard deviations of all tocol isomers of three main important pharmacokinetic parameters,  $AUC_{0-10}$ ,  $C_{max}$  and  $T_{max}$  were estimated by plasma mean concentrations using GraphPad Prism 5 and PKSolver 2.0 programs, which gave identical results without indicating the variation within groups. The pharmacokinetic analyses of plasma concentrations were carried out at the time intervals of each subject in each group by PKSolver 2.0, which



**Figures 2A and 2B: A typical HPLC elution profile of plasma sample on normal phase silica column after administration of single dose of 125 mg, 250 mg, or 500 mg of DeltaGold-based  $\delta$ -tocotrienol:** There were no peaks of  $\delta$ -tocotrienol and  $\delta$ -tocopherol of all plasma samples of 125 mg participants at 0 h and 1 h (only  $\alpha$ -,  $\beta$ -,  $\gamma$ -tocopherol, and  $\beta$ -tocotrienol). Small peak of  $\delta$ -tocotrienol appeared after 2 h, and reached maximum 3 h, small peak of  $\delta$ -tocopherol was also appeared at 3 h (Figure 2A), then started declining the levels of  $\delta$ -tocopherol and  $\delta$ -tocotrienol after 4 h -10 h (Figure 2B). Similar patterns of HPLC profile was noted throughout HPLC analyses of all samples (Figures 2A and 2B).

A. Subjects # 1 - 11 (125 mg).								
Tocols----->	$\delta$ -tocotrienol	$\gamma$ -tocotrienol	$\beta$ -tocotrienol	$\alpha$ -tocotrienol	$\delta$ -Tocopherol	$\gamma$ -Tocopherol	$\beta$ -Tocopherol	$\alpha$ -Tocopherol
Hour	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
0 h.			206 ± 34			312 ± 20	513 ± 79	1415 ± 66'
1 h.		36 ± 8	442 ± 42	123 ± 10		362 ± 36	570 ± 64	1487 ± 78
2 h.	151 ± 57	118 ± 32	488 ± 58	127 ± 8	134 ± 31	387 ± 29	704 ± 61	1521 ± 32
3 h.	829 ± 24	281 ± 21	681 ± 34	139 ± 11	229 ± 29	408 ± 34	809 ± 79	1554 ± 49
4 h.	659 ± 70	208 ± 34	979 ± 80	10 ± 10	240 ± 29	450 ± 71	887 ± 87	1610 ± 62
6 h.	122 ± 34	124 ± 21	543 ± 57	88 ± 13	341 ± 62	508 ± 25	956 ± 70	1822 ± 48
8 h.	102 ± 30	91 ± 18	493 ± 68	47 ± 10	210 ± 70	203 ± 31	315 ± 39	1194 ± 71
10 h.	47 ± 21	68 ± 21	311 ± 47	24 ± 9	142 ± 20	155 ± 15	200 ± 18	1038 ± 65
Total ng/ml	1910	953	4142	650	1296	2785	4954	11641
# of samples	6	7	8	7	6	8	8	8
B. Subjects # 12 - 22 (250 mg)								
Tocols----->	$\delta$ -tocotrienol	$\gamma$ -tocotrienol	$\beta$ -tocotrienol	$\alpha$ -tocotrienol	$\delta$ -Tocopherol	$\gamma$ -Tocopherol	$\beta$ -Tocopherol	$\alpha$ -Tocopherol
Hour	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
0 h.			231 ± 38			370 ± 27	523 ± 38	1598 ± 92'
1 h.	164 ± 99	113 ± 41	568 ± 50	157 ± 7	289 ± 61	455 ± 29	581 ± 64	1614 ± 109
2 h.	727 ± 100	213 ± 48	776 ± 36	173 ± 10	483 ± 66	497 ± 37	636 ± 58	1629 ± 91
3 h.	1920 ± 58	834 ± 28	998 ± 54	205 ± 9	541 ± 27	643 ± 37	949 ± 127	1719 ± 88
4 h.	854 ± 83	632 ± 62	1084 ± 82	155 ± 10	756 ± 73	407 ± 39	785 ± 92	1756 ± 60
6 h.	432 ± 46	372 ± 60	752 ± 60	141 ± 13	619 ± 36	273 ± 32	722 ± 79	1931 ± 93
8 h.	204 ± 67	356 ± 34	563 ± 55	104 ± 10	481 ± 38	238 ± 31	321 ± 33	1256 ± 103
10 h.	48 ± 24	143 ± 50	337 ± 58	44 ± 9	360 ± 16	179 ± 20	284 ± 31	1085 ± 90
Sum	4349	2683	5339	1113	3529	3062	4801	12588
Count	7	7	8	7	7	8	8	8
C. Subjects # 23 - 33 (500 mg)								
Tocols----->	$\delta$ -tocotrienol	$\gamma$ -tocotrienol	$\beta$ -tocotrienol	$\alpha$ -tocotrienol	$\delta$ -Tocopherol	$\gamma$ -Tocopherol	$\beta$ -Tocopherol	$\alpha$ -Tocopherol
Hour	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
0 h.			316 ± 64			249 ± 31	599 ± 65	1829 ± 91'
1 h.	63 ± 24	97 ± 19	578 ± 55	235 ± 8	525 ± 57	410 ± 46	676 ± 53	1879 ± 136
2 h.	661 ± 74	299 ± 29	877 ± 44	245 ± 7	723 ± 60	537 ± 48	726 ± 58	1931 ± 84
3 h.	1228 ± 79	1182 ± 61	1279 ± 116	290 ± 10	1028 ± 72	606 ± 48	1045 ± 148	1922 ± 96
4 h.	2117 ± 124	1225 ± 61	11922 ± 52	240 ± 7	628 ± 62	501 ± 40	793 ± 63	2018 ± 109
6 h.	3278 ± 61	989 ± 49	793 ± 61	183 ± 7	495 ± 62	372 ± 29	613 ± 63	2188 ± 147
8 h.	1652 ± 75	607 ± 50	631 ± 74	143 ± 6	265 ± 56	244 ± 31	387 ± 26	1723 ± 77
10 h.	579 ± 38	278 ± 51	380 ± 51	73 ± 4	236 ± 50	237 ± 35	228 ± 27	1188 ± 89
Sum	9578	4697	6046	1499	3900	3156	5067	14678
Count	7	7	8	7	7	8	8	8

Table 2: Estimation of plasma tocopherols by normal phase HPLC of pharmacokinetic human study after feeding single dose of 125 mg of  $\delta$ -tocotrienol in one day. Values represent Standard Deviation (SD).

provided values of the pharmacokinetic parameters with standard deviation (SD) and standard error (SE).

$\delta$ -tocotrienol showed dose-dependent increases in plasma area under the curve  $AUC_{0-110}$  (ng/ml) for 125 mg (2464 ± 192), 250 mg (5413 ± 274), and 500 mg (14986 ± 363) significantly different at  $P < 0.001$  from each other (Table 3). Similar increases of  $AUC_{0-110}$  (ng/ml), 2487 ± 201, 5515 ± 287, 17112 ± 445; plasma peak concentration ( $C_{max}$ , ng/ml), 829 ± 24, 1920 ± 58, 3278 ± 61; time to achieve plasma peak ( $T_{max}$ , h), 3, 3, 4; elimination of half-life time ( $t_{1/2}$ , h), 1.74 ± 0.36, 1.39 ± 0.22, 2.54 ± 0.05 h; time of clearance (Cl-T, h), 0.049 ± 0.004, 0.045 ± 0.002, 0.030 ± 0.001; apparent volume of distribution ( $V_d$ , ml), 0.119 ± 0.035, 0.114 ± 0.011, 0.113 ± 0.102; and elimination rate constant ( $k_e$ ; h<sup>-1</sup>), 0.412 ± 0.059, 0.401 ± 0.039, 0.265 ± 0.028 for doses of 125 mg, 250 mg, 500 mg, respectively were observed as shown in Table 3A. Most of them were significantly different at  $P < 0.001$  (Table 3A).

A similar trend of increases of all the parameters were observed for doses of 125 mg, 250 mg, 500 mg, respectively, when measuring  $\gamma$ -tocotrienol with an  $AUC_{0-110}$  (ng/ml) of 1258 ± 126, 5413 ± 274, 6897 ± 160,  $P < 0.001$ , for  $\beta$ -tocotrienol with an  $AUC_{0-110}$  (ng/ml) of 6934 ± 130, 7080 ± 207, 7680 ± 273,  $P < 0.001$ , and for  $\alpha$ -tocotrienol with an  $AUC_{0-110}$  (ng/ml) of 870 ± 44, 1370 ± 26, 1900 ± 46,  $P < 0.001$  (table 3B, C, D). The plasma peak concentrations (ng/ml) were 281 ± 32, 834 ± 28, 1224 ± 61 for  $\gamma$ -tocotrienol, 979 ± 80, 1084 ± 82, 1279 ± 116 for  $\beta$ -tocotrienol, and 140 ± 11, 205 ± 10, 290 ± 10 for  $\alpha$ -tocotrienol at doses of 125 mg, 250 mg, 500 mg, respectively (Tables 3B-3D and Figures 2A-2D). The time to achieve plasma maximum peaks ( $T_{max}$ , h) for  $\gamma$ -tocotrienol were 3 h for both 125 mg and 250 mg doses and 4 h for the 500 mg dose (Figure 2), 4 h for both the 125 mg and 250 mg dose and 3 h for the 500 mg dose for  $\beta$ -tocotrienol, and 3 h for all three doses for  $\alpha$ -tocotrienol (Tables 3A-D and Figure 2A-D). The highest values of AUC (2464, 5412, 15567 ng/ml) and  $C_{max}$  (829, 1920, 3278 ng/

A. Subjects # 1 - 11 (125 mg)							
		$\delta$ -tocotrienol			$\gamma$ -tocotrienol		
A:	125 mg	250 mg	500 mg	B:	125 mg	250 mg	500 mg
1. Area Under Curve-0-10 ( $AUC_{0-10}$ ; ng/ml)	2463.91 $\pm$ 191.62 <sup>a</sup>	5412.50 $\pm$ 274.04 <sup>b</sup>	14985.73 $\pm$ 362.63 <sup>c</sup>		1258.18 $\pm$ 126.26 <sup>a</sup>	5412.50 $\pm$ 274.04 <sup>b</sup>	6895.96 $\pm$ 159.49 <sup>c</sup>
2. Area Under Curve-0- $\infty$ ( $AUC_{0-\infty}$ ; ng/ml)	2586.41 $\pm$ 201.01 <sup>a</sup>	5514.75 $\pm$ 287.01 <sup>b</sup>	17111.94 $\pm$ 444.71 <sup>c</sup>		1647.95 $\pm$ 270.72 <sup>a</sup>	5514.75 $\pm$ 287.01 <sup>b</sup>	7818.82 $\pm$ 397.38 <sup>c</sup>
3. Plasma Peak Concentration ( $C_{max}$ ; ng/ml)	828.82 $\pm$ 24.28 <sup>a</sup>	1920.36 $\pm$ 57.99 <sup>b</sup>	3278.00 $\pm$ 124.13 <sup>c</sup>		281.34 $\pm$ 21.22 <sup>a</sup>	833.73.36 $\pm$ 28.22 <sup>b</sup>	1224.64 $\pm$ 61.28 <sup>c</sup>
4. Time to achieve plasma peak ( $T_{max}$ ; h)	3	3	4		3	3	3.55 $\pm$ 0.52
5. Elimination of Half-life time ( $t_{1/2}$ ; h)	1.74 $\pm$ 0.36 <sup>a</sup>	1.39 $\pm$ 0.22 <sup>a</sup>	2.54 $\pm$ 0.05 <sup>b</sup>		3.82 $\pm$ 0.99 <sup>a</sup>	1.39 $\pm$ 0.28 <sup>b</sup>	2.25 $\pm$ 0.32 <sup>c</sup>
6. Time of clearance (Cl-T; l/h)	0.049 $\pm$ 0.004 <sup>a</sup>	0.045 $\pm$ 0.002 <sup>a</sup>	0.030 $\pm$ 0.001 <sup>b</sup>		0.078 $\pm$ 0.012 <sup>a</sup>	0.045 $\pm$ 0.002 <sup>a</sup>	0.061 $\pm$ 0.010 <sup>b</sup>
7. Apparent volume of distribution (Vd/f)	0.179 $\pm$ 0.035 <sup>a</sup>	0.114 $\pm$ 0.011 <sup>b</sup>	0.613 $\pm$ 0.102 <sup>c</sup>		0.553 $\pm$ 0.084 <sup>a</sup>	0.461 $\pm$ 0.114 <sup>a</sup>	0.635 $\pm$ 0.060 <sup>b</sup>
8. Elimination rate constant ( $K_e$ ; h <sup>-1</sup> )	0.381 $\pm$ 0.059 <sup>a</sup>	0.401 $\pm$ 0.039 <sup>b</sup>	0.050 $\pm$ 0.008 <sup>c</sup>		0.113 $\pm$ 0.026 <sup>a</sup>	0.133 $\pm$ 0.037 <sup>a</sup>	0.097 $\pm$ 0.021 <sup>b</sup>
		$\beta$ -tocotrienol			$\alpha$ -tocotrienol		
C:	125 mg	250 mg	500 mg	D:	125 mg	250 mg	500 mg
1. Area Under Curve-0 - 10 ( $AUC_{0-10}$ ; ng/ml)	6933.73 $\pm$ 129.58 <sup>a</sup>	7080.36 $\pm$ 206.62 <sup>a</sup>	7680.41 $\pm$ 272.59 <sup>b</sup>		869.96 $\pm$ 43.95 <sup>a</sup>	1369.91 $\pm$ 26.30 <sup>b</sup>	1900.68 $\pm$ 46.29 <sup>c</sup>
2. Area Under Curve-0 - $\infty$ ( $AUC_{0-\infty}$ ; ng/ml)	8839.28 $\pm$ 656.29 <sup>a</sup>	9184.14 $\pm$ 674.76 <sup>a</sup>	10391.37 $\pm$ 621.69 <sup>b</sup>		1041.77 $\pm$ 108.82 <sup>a</sup>	1558.09 $\pm$ 77.13 <sup>b</sup>	22290.14 $\pm$ 65.53 <sup>c</sup>
3. Plasma Peak Concentration ( $C_{max}$ ; ng/ml)	979.00 $\pm$ 79.45 <sup>a</sup>	1083.73 $\pm$ 82.26 <sup>b</sup>	1279.00 $\pm$ 116.44 <sup>c</sup>		139.91 $\pm$ 11.03 <sup>a</sup>	204.91 $\pm$ 9.47 <sup>b</sup>	290.09 $\pm$ 9.84 <sup>c</sup>
4. Time to achieve plasma peak ( $T_{max}$ ; h)	4	3.64 $\pm$ 0.41	3		2.73 $\pm$ 0.65	3	3
5. Elimination of Half-life time ( $t_{1/2}$ ; h)	3.92 $\pm$ 0.86 <sup>a</sup>	3.90 $\pm$ 0.79 <sup>a</sup>	4.39 $\pm$ 0.63 <sup>a</sup>		2.62 $\pm$ 0.81 <sup>a</sup>	2.89 $\pm$ 0.84 <sup>a</sup>	3.71 $\pm$ 0.29 <sup>b</sup>
6. Time of clearance (Cl-T; l/h)	0.094 $\pm$ 0.007 <sup>a</sup>	0.090 $\pm$ 0.007 <sup>a</sup>	0.080 $\pm$ 0.005 <sup>b</sup>		0.303 $\pm$ 0.135 <sup>a</sup>	0.161 $\pm$ 0.008 <sup>b</sup>	0.109 $\pm$ 0.003 <sup>c</sup>
7. Apparent volume of distribution (Vd/f)	0.174 $\pm$ 0.011 <sup>a</sup>	0.223 $\pm$ 0.013 <sup>b</sup>	0.415 $\pm$ 0.019 <sup>c</sup>		0.590 $\pm$ 0.061 <sup>a</sup>	0.922 $\pm$ 0.067 <sup>b</sup>	1.365 $\pm$ 0.045 <sup>c</sup>
8. Elimination rate constant ( $K_e$ ; h <sup>-1</sup> )	0.542 $\pm$ 0.062 <sup>a</sup>	0.404 $\pm$ 0.032 <sup>b</sup>	0.192 $\pm$ 0.014 <sup>c</sup>		1.234 $\pm$ 0.211 <sup>a</sup>	0.201 $\pm$ 0.050 <sup>b</sup>	0.214 $\pm$ 0.009 <sup>b</sup>

**Table 3:** Pharmacokinetic parameters after feeding single dose of various concentrations of  $\delta$ -tocotrienol (125 or 250 or 500 mg) in one day.

<sup>a-c</sup>Values in a row not sharing a common letter are significantly different at P <0.001-0.01.

<sup>a</sup>Values represent Standard Deviation (SD).

ml) of all doses were found with  $\delta$ -tocotrienol, and lowest values AUC (870, 1570, 1901 ng/ml) and  $C_{max}$  (140, 205, 290 ng/ml) were found with  $\alpha$ -tocotrienol. The time to elute maximum peak occurred between 3 h to 6 h (Tables 3A-C).

The impact of  $\gamma$ -,  $\beta$ -, and  $\alpha$ -tocotrienol were also determined on half-life time ( $t_{1/2}$ ; h), time of clearance (Cl-T, Cl/h), volume of distribution (Vd, ml), and elimination rate constant ( $K_e$ ; h<sup>-1</sup>), respectively, for doses of 125 mg, 250 mg and 500 mg (Tables 3B-D). The values for these parameters showed dose-dependent decreases, which were mostly significantly different at P<0.01 as reported in Tables 3A-D.

For  $\delta$ -tocopherol, the plasma values of  $AUC_{10-110}$  (1971  $\pm$  197, 5007  $\pm$  164, 5120  $\pm$  268, ng/ml, P<0.001);  $AUC_{10-\infty}$  (2647  $\pm$  244, 7726  $\pm$  485, 6373  $\pm$  634, ng/ml, P<0.001; as extrapolated by PKSolver 2.0); and  $C_{max}$  (341  $\pm$  62, 756  $\pm$  72, 1028  $\pm$  72, ng/ml, P<0.001) also showed dose-dependent increases as reported in Table 4A, and  $T_{max}$  was 6, 4, and 3 h. Similarly, for  $\gamma$ -tocopherol these main pharmacokinetic parameters,  $AUC_{10-110}$  (3565, 3576, 3898 ng/ml);  $AUC_{10-\infty}$  (4118, 4913, 5638 ng/ml);  $C_{max}$  (508, 643, 606 ng/ml) showed significant dose-dependent increases (P<0.001), and  $T_{max}$  was 6, 3, and 3 h for doses of 125 mg, 250 mg, 500 mg, respectively (Table 4B). For  $\beta$ -tocopherol,  $AUC_{10-110}$  (6410, 5974, 6183 ng/ml);  $AUC_{10-\infty}$  (6938, 7551, 7634 ng/ml) showed significant dose-dependent increases (P<0.01), whereas,  $C_{max}$  (956, 950, 1045 ng/ml) was significant only between doses of 125 mg and 250 mg versus 500 mg (P<0.05), and  $T_{max}$  was 5, 3, and 3 h for doses of 125 mg, 250 mg, and 500 mg, respectively (Table 4C). The maximum values were observed with  $\alpha$ -tocopherol for  $AUC_{10-110}$  (14754, 15853, 18682 ng/ml);  $AUC_{10-\infty}$  (22289, 23622, 26548 ng/ml);  $C_{max}$  (1822, 1931, 2188 ng/ml), which also showed significant dose-dependent increases (P<0.01), and  $T_{max}$  was 6 h for all three doses (Table 4D).

The effects of  $\delta$ -tocopherol,  $\gamma$ -tocopherol,  $\beta$ -tocopherol, and  $\alpha$ -tocopherol were also reported on half-life time ( $t_{1/2}$ ; h), time of clearance (Cl-T, Cl/h), volume of distribution (Vd/L), and elimination

rate constant ( $K_e$ ; h<sup>-1</sup>), respectively, for doses of 125 mg, 250 mg and 500 mg (Tables 4A-D). The values for these parameters showed dose-dependent decreases, which were significantly different with P<0.01 as reported in Tables 3A-D. Interestingly, the values of Vd (ml) showed dose-dependent increases with all four tocopherol isomers (Tables 4A-D). The time to achieve peak plasma maximum ( $T_{max}$ ; h) varied between 3 h to 4 h for isomers of tocotrienols and 3 h to 6 h for isomers of tocopherols for all three doses (Tables 3A-3D and 4A-4D). The summary of plasma concentrations (ng/ml) based on Tables 3 and 4 A, B, C, D of all four tocotrienols and tocopherols were reported in Table 5. The highest plasma concentrations were obtained for  $\alpha$ -tocopherol as shown in Table 5 and Figure 3.

### Effects of $\delta$ -tocotrienol on human plasma cytokines/proteins and microRNAs

We have reported earlier the influence of  $\delta$ -tocotrienol on several key cytokines/proteins involved in various diseases [5, 6]. In the present paper, only those plasma cytokines/proteins are described which are important for inflammation, cardiovascular disease, cancer, and aging. The values of each cytokine/protein were based on percentages of pre-dose values of 0 h plasma samples of 125 mg and 500 mg  $\delta$ -tocotrienol (regarded as 100%) versus post-dose values of  $\delta$ -tocotrienol at 3 h (125 mg dose), 3 h (250 mg dose) and 6 h (500 mg dose) plasma samples, respectively. The importance of these 11 cytokines/proteins is reported in Table 6.

### Effects of annatto-based $\delta$ -tocotrienol on plasma miRNAs (miRNAs)

Recently, microRNAs (miRNAs) were discovered to play an important role in various diseases like cancer, diabetes, cardiovascular diseases and neurological disorders. Plasma samples from 0 h and 3h (125 mg dose) and 0 h and 6 h (500 mg dose) of  $\delta$ -tocotrienol were analyzed for miRNA analysis. miR-34a which is normally found to be

A:	$\delta$ -tocopherol			B:	$\gamma$ -tocopherol		
	125 mg	250 mg	500 mg		125 mg	250 mg	500 mg
1. Area Under Curve-0 - 10 ( $AUC_{0-10}$ ; ng/ml)	1971.00 $\pm$ 197.23 <sup>a</sup>	5007.05 $\pm$ 164.16 <sup>b</sup>	5119.68 $\pm$ 268.21 <sup>b</sup>	3564.64 $\pm$ 136.64 <sup>a</sup>	3575.96 $\pm$ 154.98 <sup>a</sup>	3898.41 $\pm$ 130.37 <sup>b</sup>	
2. Area Under Curve-0 - $\infty$ ( $AUC_{0-\infty}$ ; ng/ml)	2647.23 $\pm$ 243.57 <sup>a</sup>	7726.83 $\pm$ 484.561 <sup>b</sup>	6373.12 $\pm$ 633.37 <sup>c</sup>	4117.63 $\pm$ 205.74 <sup>a</sup>	4912.99 $\pm$ 374.53 <sup>b</sup>	5638.22 $\pm$ 616.15 <sup>c</sup>	
3. Plasma Peak Concentration ( $C_{max}$ ; ng/ml)	341.18 $\pm$ 62.19 <sup>a</sup>	756.27 $\pm$ 72.70 <sup>b</sup>	1027.73 $\pm$ 71.93 <sup>c</sup>	507.64 $\pm$ 24.68 <sup>a</sup>	643.36 $\pm$ 37.26 <sup>b</sup>	605.64 $\pm$ 48.17 <sup>b</sup>	
4. Time to achieve plasma peak ( $T_{max}$ ; h)	6	4.18 $\pm$ 0.60	3	5.46 $\pm$ 0.93	3	2.82 $\pm$ 0.41	
5. Elimination of Half-life time ( $t_{1/2}$ ; h)	3.25 $\pm$ 0.79 <sup>a</sup>	5.22 $\pm$ 0.62 <sup>b</sup>	3.58 $\pm$ 0.67 <sup>a</sup>	2.45 $\pm$ 0.37 <sup>a</sup>	5.17 $\pm$ 1.06 <sup>b</sup>	4.99 $\pm$ 0.88 <sup>b</sup>	
6. Time of clearance (Cl-T; l/h)	0.048 $\pm$ 0.004 <sup>a</sup>	0.032 $\pm$ 0.002 <sup>b</sup>	0.079 $\pm$ 0.007 <sup>c</sup>	0.038 $\pm$ 0.026 <sup>a</sup>	0.051 $\pm$ 0.004 <sup>b</sup>	0.086 $\pm$ 0.018 <sup>c</sup>	
7. Apparent volume of distribution (Vd/f)	0.688 $\pm$ 0.133 <sup>a</sup>	0.431 $\pm$ 0.050 <sup>b</sup>	0.436 $\pm$ 0.036 <sup>b</sup>	0.209 $\pm$ 0.021 <sup>a</sup>	0.350 $\pm$ 0.020 <sup>b</sup>	0.683 $\pm$ 0.041 <sup>c</sup>	
8. Elimination rate constant ( $K_e$ ; h <sup>-1</sup> )	0.118 $\pm$ 0.032 <sup>a</sup>	0.076 $\pm$ 0.008 <sup>b</sup>	0.183 $\pm$ 0.027 <sup>c</sup>	0.184 $\pm$ 0.121 <sup>a</sup>	0.146 $\pm$ 0.013 <sup>b</sup>	0.126 $\pm$ 0.026 <sup>a</sup>	
C:	$\beta$ -tocopherol			D:	$\alpha$ -tocopherol		
	125 mg	250 mg	500 mg		125 mg	250 mg	500 mg
1. Area Under Curve-0 - 10 ( $AUC_{0-10}$ ; ng/ml)	6410.18 $\pm$ 195.55 <sup>a</sup>	5973.77 $\pm$ 403.98 <sup>a</sup>	6182.55 $\pm$ 195.67 <sup>a</sup>	14754.27 $\pm$ 218.40.21 <sup>a</sup>	15852.50 $\pm$ 518.04 <sup>a</sup>	18681.86 $\pm$ 600.04 <sup>b</sup>	
2. Area Under Curve-0 - $\infty$ ( $AUC_{0-\infty}$ ; ng/ml)	6937.76 $\pm$ 198.62 <sup>a</sup>	7550.96 $\pm$ 495.54 <sup>a,b</sup>	7633.65 $\pm$ 393.05 <sup>b</sup>	22288.86 $\pm$ 1504.88.69 <sup>a</sup>	23622.40 $\pm$ 2044.09 <sup>a,b</sup>	26547.56 $\pm$ 1429.60 <sup>b</sup>	
3. Plasma Peak Concentration ( $C_{max}$ ; ng/ml)	956.09 $\pm$ 70.06 <sup>a</sup>	949.91 $\pm$ 126.37 <sup>a</sup>	1045.09 $\pm$ 147.81 <sup>a</sup>	1822.00 $\pm$ 48.24 <sup>a</sup>	1931.00 $\pm$ 92.54 <sup>b</sup>	2188 $\pm$ 147.61 <sup>c</sup>	
4. Time to achieve plasma peak ( $T_{max}$ ; h)	5.18 $\pm$ 1.17	3.09 $\pm$ 0.30	3.09 $\pm$ 0.32	6	6	5.46 $\pm$ 0.93	
5. Elimination of Half-life time ( $t_{1/2}$ ; h)	1.82 $\pm$ 0.23 <sup>a</sup>	3.97 $\pm$ 0.66 <sup>a</sup>	3.27 $\pm$ 0.61 <sup>a</sup>	4.99 $\pm$ 0.69 <sup>a</sup>	4.91 $\pm$ 0.84 <sup>a</sup>	3.58 $\pm$ 0.67 <sup>a</sup>	
6. Time of clearance (Cl-T; l/h)	0.119 $\pm$ 0.003 <sup>a</sup>	0.110 $\pm$ 0.007 <sup>b</sup>	0.112 $\pm$ 0.006 <sup>b</sup>	0.017 $\pm$ 0.015 <sup>a</sup>	0.011 $\pm$ 0.001 <sup>b</sup>	0.019 $\pm$ 0.001 <sup>a</sup>	
7. Apparent volume of distribution (Vd/f)	0.096 $\pm$ 0.006 <sup>a</sup>	0.227 $\pm$ 0.026 <sup>b</sup>	0.410 $\pm$ 0.035 <sup>c</sup>	0.070 $\pm$ 0.002 <sup>a</sup>	0.127 $\pm$ 0.004 <sup>b</sup>	0.232 $\pm$ 0.010 <sup>c</sup>	
8. Elimination rate constant ( $K_e$ ; h <sup>-1</sup> )	1.247 $\pm$ 0.073 <sup>a</sup>	0.489 $\pm$ 0.063 <sup>b</sup>	0.275 $\pm$ 0.023 <sup>c</sup>	0.237 $\pm$ 0.217 <sup>a</sup>	0.084 $\pm$ 0.008 <sup>ab</sup>	0.081 $\pm$ 0.006 <sup>b</sup>	

**Table 4:** Pharmacokinetic parameters after feeding single dose of various concentrations of  $\delta$ -tocotrienol (125, or 250, or 500 mg) in one day.

<sup>a-c</sup>Values in a row not sharing a common letter are significantly different at  $P < 0.001 - 0.01$ .

<sup>a</sup>Values represent Standard Deviation (SD).

A:	$\delta$ -tocotrienol			B:	$\delta$ -Tocopherol		
	125 mg	250 mg	500 mg		125 mg	250 mg	500 mg
1. Total sum Pre-dose treatment at 0 hr (ng/ml)	0	0	0	0	0	0	
2. Total sum Post-dose treatment (ng/ml)	1910	4349	9578	1296	3529	3900	
C:	$\gamma$ -tocotrienol			D:	$\gamma$ -Tocopherol		
	125 mg	250 mg	500 mg		125 mg	250 mg	500 mg
1. Total sum Pre-dose treatment at 0 hr (ng/ml)	0	0	0	312	370	249	
2. Total sum Post-dose treatment (ng/ml)	926	2663	4677	2785	3062	3156	
E:	$\beta$ -tocotrienol			F:	$\beta$ -Tocopherol		
	125 mg	250 mg	500 mg		125 mg	250 mg	500 mg
1. Total sum Pre-dose treatment at 0 hr (ng/ml)	206	231	316	513	523	599	
2. Total sum Post-dose treatment (ng/ml)	4142	5339	6046	4954	4801	5067	
G:	$\alpha$ -tocotrienol			H:	$\alpha$ -Tocopherol		
	125 mg	250 mg	500 mg		125 mg	250 mg	500 mg
1. Total sum Pre-dose treatment at 0 hr (ng/ml)	0	0	0	1415	1598	1829	
2. Total sum Post-dose treatment (ng/ml)	558	979	1409	11641	12588	14678	

**Table 5:** Impact of annatto-based  $\delta$ -tocotrienol of various doses on plasma tocopherols concentrations in humans.

Subjects # 1 - 11 (125 mg), 12 - 22 (250 mg), 23 - 33 (500 mg).

increased during bipolar disorder, was downregulated by  $\delta$ -tocotrienol feeding (Table 7), whereas the expression of miR-107, miR-122a, and miR-132, whose levels are low in Alzheimer's patients, were up-regulated by  $\delta$ -tocotrienol treatment (Table 7).

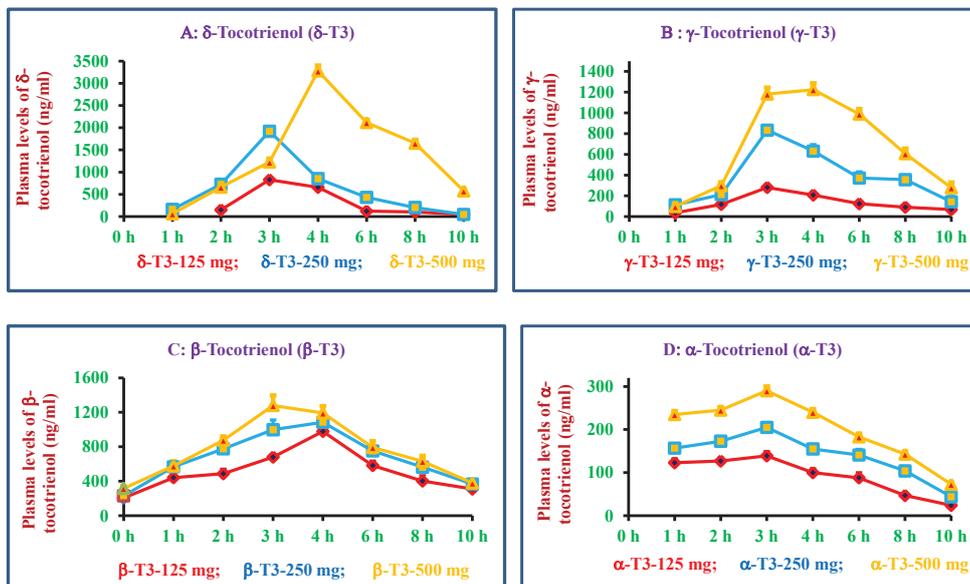
## Discussion

The present study described the pharmacokinetics and bioavailability of various doses of annatto-based  $\delta$ -tocotrienol (without  $\alpha$ -tocopherol) in thirty three ( $n=33$ ) well fed healthy participants. This is the first study to report the effects of  $\delta$ -tocotrienol on the pharmacokinetic parameters of all eight isomers of the vitamin E family ( $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienols and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherols). The bioavailability of  $\delta$ -tocotrienol resulted in dose-dependent increases of plasma  $AUC_{0-10}$ ,  $AUC_{0-\infty}$ ,  $C_{max}$ , and  $T_{max}$  that varies between 3 h-4 h for isomers of tocotrienols and 3 h-6 h for isomers of tocopherols at 125 mg, 250 mg, 500 mg doses. The  $t_{1/2}$  (h) of these three main parameters peaked from 1.39 h to 4.39 h for the tocotrienol group (Table 3) as compared to 1.82 h to 5.22 h for the tocopherol group (Table 4), indicating a longer time of excretion for tocopherols compared to tocotrienols which further supports that

tocotrienols have better bioavailability than tocopherols as reported recently [26].

The effects of all isomers of tocopherols were also reported on Cl-T, Cl/h, Vd, and  $K_e$ ; h<sup>-1</sup> for doses of 125 mg, 250 mg and 500 mg. These parameters were significantly different ( $P < 0.001 - 0.01$ ) from each other for all three doses. After administering doses of 750 mg and 1000 mg of  $\delta$ -tocotrienol under the same conditions, higher dose-dependent increases for  $AUC_{0-10}$  and  $C_{max}$  were observed than for the 125 mg, 250 mg, and 500 mg doses (data is not shown). These results confirmed the preliminary findings of pharmacokinetics of  $\delta$ -tocotrienol reported in pancreatic cancer patients, where  $\delta$ -tocotrienol was well tolerated at doses up to 800 mg daily without toxicity [23].

Most of the previous studies on pharmacokinetics of tocotrienols were carried out by using tocotrienol mixtures containing  $\alpha$ -tocopherol. It was indicated that the bioavailability of  $\alpha$ -tocotrienol is significantly affected due to the presence of  $\alpha$ -tocopherol in the mixture [18]. Moreover, bio-discrimination between tocopherols in humans reduces the rate of tocotrienol absorption, and thus the desirable physiological



**Figures 3A, B, C, D: Estimation of plasma peak concentration ( $C_{max}$ , ng/ml) of  $\delta$ -,  $\gamma$ -,  $\beta$ -,  $\alpha$ -tocotrienol of various doses:** The single dose of 125 mg, 250 mg, or 500 mg of DeltaGold-based  $\delta$ -tocotrienol was administered in one day to well-fed healthy subject (11/dose). The blood samples were collected in ethylene diamine tetra acetic acid (EDTA) glazed tubes at pre-dose (0 h) to post-dose 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h intervals of each subject. The plasma samples were harvested and processed to carry out normal phase HPLC analyses of each subject as described in Material and Method section. Values are means  $\pm$  standard deviation ( $n = 11$ /dose). Values are significantly different at  $P < 0.001$  from each other.

#	Cytokines	$\delta$ -tocotrienol						Description	Functions
		0-h	3-h	0-h	3-h	0-h	6-h		
		125 mg/day	250 mg/day	500 mg/day					
<b>I Cytokines involved in inflammation</b>									
1	GM-CSF	100	89	100	71	100	44	Granulocyte Macrophage Colony Stimulating Factor	It plays a key role in inflammatory and autoimmune disease
2	IL-13	100	76	100	66	100	42	Interleukin-13	Key role in the activities of natural killer cells and T lymphocytes.
3	Eotaxin-3	100	87	100	67	100	71	Chemokine ligand 26	It activates eosinophils, basophils and Th2 type T lymphocytes, stimulated with interleukin 4.
4	PIGF-1	100	67	100	58	100	55	Plasmenogen Activator Inhibitor-1	A multitask cytokine involed in various types of inflammation.
5	G-CSF	100	88	100	81	100	40	Granulocyte Colony Stimulating Factor	Stimulates proliferation, differentiation, function of neutrophil precursor.
6	Rantes	100	76	100	71	100	39	Regulated upon activation normal T-cell expressed	It induces proliferation/activation of certain natural-killer cells, also an HIV-suppressive factor.
<b>II Cytokines involved in cardiovascular diseases</b>									
7	$\beta$ -NGF	100	78	100	73	100	56	$\beta$ -Nerve Growth Factor	Exerts a variety of effects in the cardiovascular system and on endothelial cells.
8	Adipo	100	189	100	261	100	289	Adiponectin (GBP-28)	Adipocyte-secreted insulin-sentizing and anti-atherosclrotic hormone. Its low level resulted decline in insulin sensitivity in humans.
<b>III Cytokines involved in cancer</b>									
9	SCF	100	84	100	86	100	82	Stem Cell Factor	Tumor-induced brain injury/brain cell-mediated SCF expression leads to tumor growth.
10	TGF- $\beta$ 1	100	146	100	167	100	734	Tumor growth Factor- $\beta$ 1	Cytokine controls proliferation, adhesion, migration & other functions in many cells.
<b>IV Cytokines involved in ageing</b>									
11	Leptin	100	193	100	289	100	341	Leptin	Regulating energy intake/energy expenditure by decreasing appetite/increased metabolism

**Table 6:** Cytokines of plasma samples of pharmacokinetic of  $\delta$ -tocotrienol-125 mg-0 h versus 3 h; 250 mg-0 h versus 3 h; or 500 mg-0 h versus 6 h in one day in humans.

	miRNA	0-h (125 mg) Percentage	3-h (125 mg) Percentage	0-h (500 mg) Percentage	6-h (500 mg) Percentage
<b>A</b>	<b>Inflammation</b>				
1	miR-9	100	88	100	44
2	miR-34a	100	72	100	59
3	miR-107	100	156	100	173
4	miR122a	100	166	100	196
5	miR-132	100	199	100	145
6	miR-148a	100	208	100	233
7	miR-181a	100	48	100	21
<b>B</b>	<b>Cardiovascular</b>				
8	miR-24	100	77	100	45
9	miR-19b	100	91	100	70
<b>C</b>	<b>Cancer</b>				
10	miR-1	100	78	100	63
11	miR-7	100	94	100	85
12	miR-15b	100	110	100	132
13	miR-17-5p	100	106	100	168
14	miR-19a	100	95	100	36
15	miR-26a	100	80	100	62
16	miR-106a	100	74	100	56
17	miR-143	100	63	100	36
18	miR-145	100	54	100	44
19	miR-182	100	76	100	64
20	miR-192	100	28	100	21
21	miR-194	100	50	100	21
22	miR-196a	100	65	100	43
23	miR-199a	100	81	100	65
24	miR-204	100	45	100	41
25	miR-205	100	39	100	45
26	miR-222	100	55	100	51
27	miR-342	100	70	100	52

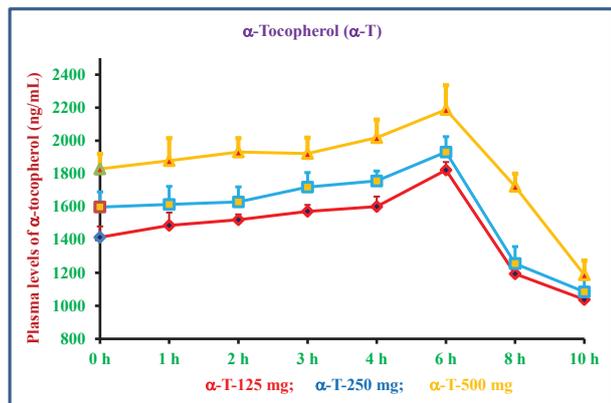
**Table 7:** Plasma miRNAs of  $\delta$ -tocotrienol at 0 h - 3 h (125 mg) and 0 h - 6 h (500 mg) of pharmacokinetic study in humans.

effects of tocotrienol may not be achieved [28]. In rats, small amounts of orally administered  $\alpha$ -tocopherol were found to have greater effects on  $\alpha$ -tocotrienol serum concentrations [28]. In this study, 8 h of feeding tocotrienol mixture plus  $\alpha$ -tocopherol (10 mg  $\alpha$ -tocotrienol + 14 mg  $\gamma$ -tocotrienol +  $\alpha$ -tocopherol 1 or 10 mg) resulted in significant reduction of plasma total  $\alpha$ -tocotrienol concentration by 60% and 90% compared with the same mixture without  $\alpha$ -tocopherol. It was also reported that bio-discrimination occurs in the absorption of various tocotrienols and they were absorbed in the order of  $\alpha$ -tocotrienol >  $\gamma$ -tocotrienol >  $\delta$ -tocotrienol [10].

Further improvement in oral bioavailability and pharmacokinetics of tocotrienols was achieved by using novel self-emulsifying drug delivery systems (SEDDS), which resulted in 2.5-4.5 times higher plasma tocotrienol ( $C_{max}$ ) and also enhanced plasma tocotrienol (AUC) [24]. For example, in an oral bioavailability study in rats, an SEDDS of  $\delta$ - +  $\gamma$ -tocotrienol from annatto was up to seven-fold more bioavailable than the same tocotrienol mixture without SEDDS [32]. This study, however, showed that as the concentration of synthetic emulsifiers increased, the bioavailability of tocotrienols decreased, hence demonstrating nonlinear kinetics. A new formulation of  $\gamma$ -tocotrienol (75%) +  $\delta$ -tocotrienol (25%) versus palm oil tocotrienol-rich fraction (TRF with similar percentages of  $\gamma$ - +  $\delta$ -tocotrienol) tested in humans indicated better bioavailability for  $\gamma$ -tocotrienol than  $\delta$ -tocotrienol and TRF [33], although the study failed to test  $\gamma$ -tocotrienol and

$\delta$ -tocotrienol separately versus a mixture of the same tocotrienols and TRF from palm oil [33]. Meganathan *et al.* reported the HPLC profiles of only standard compounds but not of experimental plasma samples, and it was not clear from their data whether any other tocotrienes eluted from experimental samples, particularly  $\alpha$ -tocopherol isomer [33].

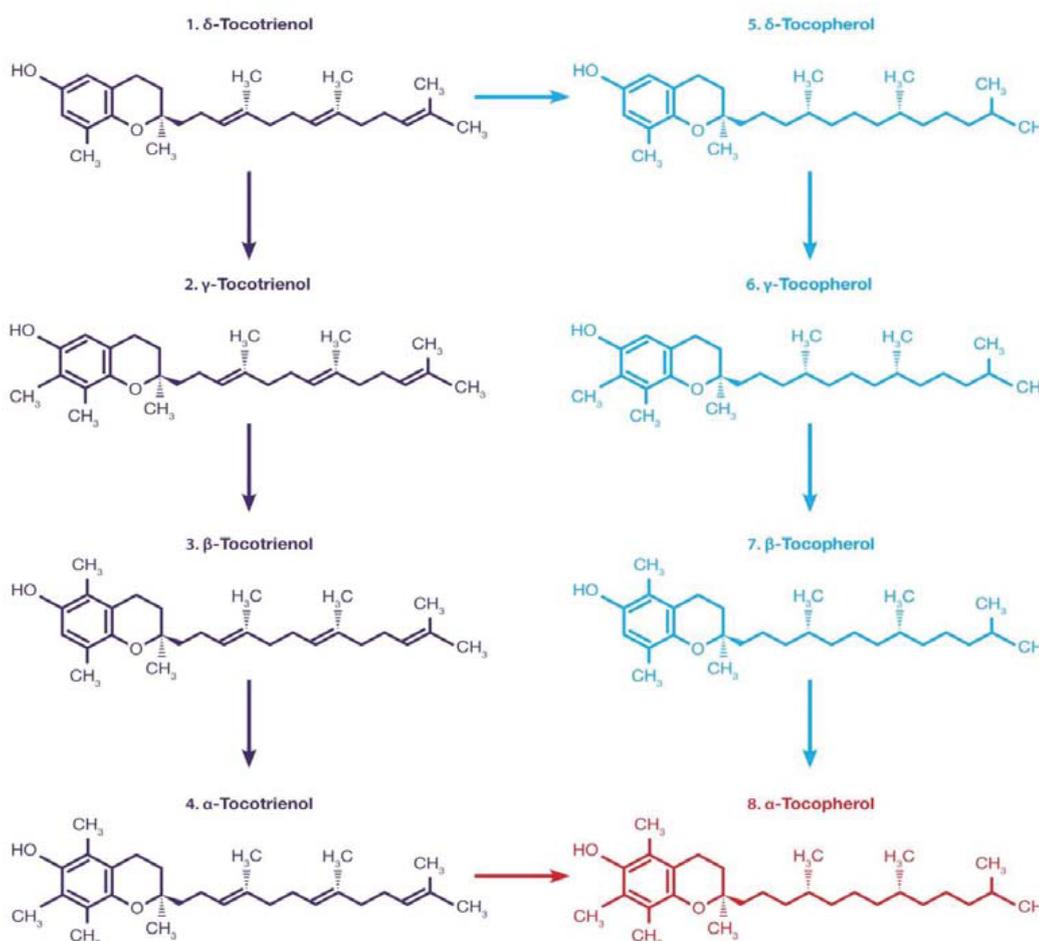
It was reported that average time to reach the highest maximum concentration ( $t_{max}$ ) of  $\alpha$ -tocopherol peaked later in both  $\gamma$ -tocotrienol and  $\alpha$ -tocopherol groups [34]. In this study, the first step in the absorption of tocotrienes is mainly attributed to passive diffusion taking place in the intestine [34]. The oral administration of  $\gamma$ -tocotrienol or  $\alpha$ -tocopherol (10 mg/kg diet) to rats resulted in plasma  $\gamma$ -tocotrienol concentrations peaking considerably earlier (2.4 h) than the plasma concentrations of  $\alpha$ -tocopherol (9.5 h), although  $\alpha$ -tocopherol had a higher overall intestinal permeability and absorption rate [34]. The rapid disappearance of tocotrienes in plasma (causing the early tocotrienol plasma  $T_{max}$  in comparison to that of  $\alpha$ -tocopherol) is also thought to be due to its preferential utilization in humans [11]. These findings are consistent with those of the present and other studies, as plasma tocotrienes peaked earlier than plasma tocopherols [10,11]. However, several relevant papers presenting human pharmacokinetic results indicated a  $T_{max}$  of up to 5 h for  $\alpha$ -tocotrienol and  $\gamma$ -tocotrienol [10, 11, 27]. This  $T_{max}$  is independent of the fed or fasted food status [27]. Our present results also supported all these findings ( $T_{max}$  3 h-4 h for  $\delta$ -,  $\gamma$ -,  $\beta$ -tocotrienols compared  $T_{max}$  for  $\alpha$ -tocopherol 6 h).



**Figure 4: Estimation of plasma peak concentration ( $C_{max}$ , ng/ml) of  $\alpha$ -tocopherol of various doses:** The doses of 125 mg, 250 mg, 500 mg/d of DeltaGold-based  $\delta$ -tocotrienol were administered to well fed healthy participants (11 subjects/dose). The blood samples were collected in ethylene diamine tetra acetic acid (EDTA) glazed tubes at pre-dose (0 h) to post-dose 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h intervals of each subject. The plasma samples were harvested and processed to carry out normal phase HPLC analyses as described in Material and Method section. Values are means  $\pm$  standard deviation (n = 11/ dose). Values are significant different at  $P < 0.01$  from each other.

This is the first report to describe the quantitative determination of all eight isomers of tocots (four tocotrienols and four tocopherols) separated from human plasma samples at 0 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, and 10 h after administering either 125 mg, or 250 mg, or 500 mg doses of annatto-based  $\delta$ -tocotrienol (without tocopherol). Importantly, our HPLC results indicated that after 2 h,  $\gamma$ -,  $\beta$ -,  $\alpha$ -tocotrienol and  $\delta$ -,  $\gamma$ -,  $\beta$ -,  $\alpha$ -tocopherol rapidly appeared as presented in Figure 5. It is assumed that  $\delta$ -tocotrienol was metabolically converted to these tocotrienols and tocopherols. A stepwise conversion of  $\delta$ -tocotrienol to  $\alpha$ -tocopherol may be occurring. In order to understand the importance of these findings, it would be necessary first to understand the biosynthesis pathways of tocotrienols and tocopherols (tocols) in nature.

The preferred proposed pathway for the biosynthesis of tocotrienols and tocopherols consists of a prenylation reaction with a polyprenyl phosphate that takes place on homogenistic acid, which is derived from *p*-hydroxyphenyl pyruvic acid, followed by the decarboxylation of homogenistic acid, and further attachment of the geranylgeranyl group at the position meta to the methyl group and cyclization. This leads to the formation of  $\delta$ -tocotrienol, which is the first compound synthesized in the biosynthesis pathway of tocots as described in 1971 [35]. The



**Figure 5: Stepwise conversion of  $\delta$ -tocotrienol to  $\alpha$ -tocopherol:** The delta-based  $\delta$ -tocotrienol 125 mg was administered to well-fed subjects for pharmacokinetic study. After 2 h of consumption,  $\delta$ -tocotrienol was appeared, which gives rise to  $\gamma$ -tocotrienol,  $\beta$ -tocotrienol,  $\alpha$ -tocotrienol by successive C-methylation, and further leads to successive reduction to give rise to  $\delta$ -tocopherol,  $\gamma$ -tocopherol,  $\beta$ -tocopherol and  $\alpha$ -tocopherol. The end product is  $\alpha$ -tocopherol (vitamin) as reported for biosynthesis of tocotrienols and tocopherols [35].

$\delta$ -tocotrienol then metabolized to the  $\gamma$ -,  $\beta$ -, and  $\alpha$ -tocotrienols by successive C-methylation, which leads to successive reduction to metabolize to  $\delta$ -,  $\gamma$ -,  $\beta$ -, and  $\alpha$ -tocopherol [35]. The end product of the biosynthesis pathway in nature is  $\alpha$ -tocopherol (vitamin E) [36].

The phenomena of bioconversion of tocotrienols to tocopherols have been discussed in earlier publications [4-6,29]. It was reported that failure of large doses of palm TRF and rice bran TRF25 to lower lipid parameters in hypercholesterolemic subjects maybe due to their conversion to tocopherols, because the plasma concentrations of tocopherols were 2- to 4-fold higher than tocotrienols as compared to placebo group. This conversion of tocotrienol to  $\alpha$ -tocopherol earlier was demonstrated by the conversion of radioactive  $\gamma$ -[4- $^3$ H] tocotrienol to  $\alpha$ -tocopherol. The radioactive synthetic  $\gamma$ -[4- $^3$ H]-tocotrienol was fed to chickens for 4 weeks, and serum was subjected to HPLC analysis to separate individual tocopherols and tocotrienols [29]. Radioactivity was found in  $\alpha$ -tocopherol,  $\beta$ -tocopherol,  $\gamma$ -tocopherol,  $\alpha$ -tocotrienol,  $\beta$ -tocotrienol,  $\gamma$ -tocotrienol, and not in  $\delta$ -tocopherol or  $\delta$ -tocotrienol [29]. The present study demonstrated that after supplementing  $\delta$ -tocotrienol, this first compound synthesized in nature was converted stepwise to other tocotrienols and tocopherols as presented in Figure 5.

The correlation between plasma levels of several pro-inflammatory and anti-inflammatory cytokines affected by  $\delta$ -tocotrienol treatment has been reported in our recent publications [4,5,37]. Most of the cytokines (eight out of eleven) were down-regulated in a dose-dependent manner by  $\delta$ -tocotrienol treatment, except adiponectin, tumor growth factor  $\beta$ 1 and leptin (Table 6). The key functions of all of these cytokines were reported in Table 6.

There were twenty seven miRNAs reported in Table 7. Seven were involved in inflammation, and  $\delta$ -tocotrienol treatment resulted down-regulation of miR-9, miR-34a, 181a and up-regulation of miR-107, miR-122a, miR-132, miR-148a. Further, miR-24 and miR-19b – associated with cardiovascular diseases – were down-regulated. Dysregulation of miR-34a is increased in neural development and a genetic risk factor for bipolar disorder (BD) patients.  $\delta$ -tocotrienol treatment of participants in the present study resulted in significant down-regulation of miR-34a, suggesting that this supplement may have a beneficial effect in the treatment of BD patients [38]. Expression of miR-107, miR-122a, and miR-132 decreases during the early stage of Alzheimer's disease, and disease progression is accelerated through regulation of *b*-site amyloid precursor protein-cleaving enzyme-1. These miRNAs were up-regulated by  $\delta$ -tocotrienol treatment in the present study, whereas other studies suggest that this type of up-regulation may be beneficial in Alzheimer's disease [39-41].

Another eighteen miRNAs (miR-19a, miR-26a, miR-106a, miR-182, miR-192, miR-194, miR-196a, miR-199a, miR-204, miR-205, miR-22 and miR-342) tested in this study were associated with various types of cancers, and were down-regulated by  $\delta$ -tocotrienol treatment, except miR-15b and miR-17-5p, which were up-regulated by the tocotrienol treatment. miR-15b was significantly up-regulated in cisplatin-resistant lung adenocarcinoma A549/CDDP cells compared with parental A549 cells [42]. miR-17-5p, on the other hand, was a key regulator of the G1/s phase cell cycle transition, and acted as an oncogene and a tumor suppressor in different cellular contexts [43]. In summary, key important functions of up-regulation or down-regulation of each miRNA are reported in Table 7.

## Conclusion

This study has established the pharmacokinetics of  $\delta$ -tocotrienol

that demonstrated pharmacokinetics and bioavailability of all eight isomers of tocotrienols and tocopherols in humans. Tocotrienols in general have lower  $T_{max}$  and  $t_{1/2}$  compared to tocopherols, which results in superior bioavailability of tocotrienols compared to tocopherols. This data also established that  $\delta$ -tocotrienol is converted stepwise to other tocotrienols and tocopherols. Moreover  $\delta$ -tocotrienol treatment resulted in down-regulation of eight cytokines and therefore may be useful in the treatment of bipolar disorder (BD) and Alzheimer's disease because of its beneficial effects on miRNAs involved in these diseases. The bioavailability of  $\delta$ -tocotrienol for the first time established in this study-supports this dietary supplement's use in the prevention of various age-related and chronic illnesses.

## Acknowledgment

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## Conflict of Interest

A.A. Qureshi, D.A. Khan, Shahid Saleem, N. Silswal, and N. Qureshi have no interest to declare. A.M. Trias and Barrie Tan are employees of American River Nutrition, Inc.

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