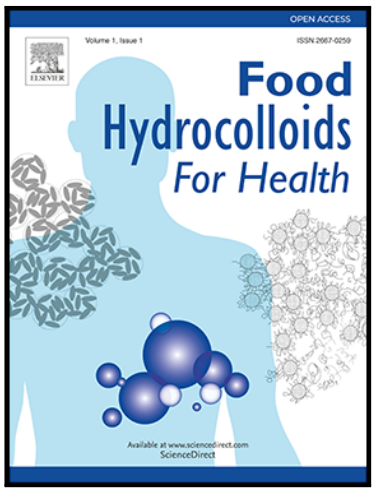

A comparative pharmacokinetic assessment of an innovative bioavailable Hydro-Oleo β-alanine complex with conventional β-alanine: A randomized double-blind, single dose, three-treatment, three-way crossover oral bioavailability study in healthy aged-human adults under fasting conditions

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A comparative pharmacokinetic assessment of an innovative bioavailable Hydro-Oleo β-alanine complex with conventional β-alanine: A randomized double-blind, single dose, three-treatment, three-way crossover oral bioavailability study in healthy aged-human adults under fasting conditions

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Abstract

High oral doses of β-alanine are often associated with uncomfortable symptoms of paresthesia, discouraging adherence to supplementation. This study aimed to evaluate the bioavailability, pharmacokinetics, and tolerability of a 400 mg Hydro-Oleo encapsulated β-alanine complex specifically designed to reduce paresthesia. A randomized, double-blind, single-dose, three-treatment, three-way crossover oral bioavailability study was conducted in healthy older adults under fasting conditions. The study compared the β-alanine complex with low (400 mg) and high (1200 mg) doses of conventional β-alanine. The β-alanine complex (400 mg) achieved a nearly 4.5-fold and 1.3-fold increase in circulating concentrations compared to 400 mg and 1200 mg of conventional β-alanine, respectively. Importantly, no adverse effects, including paresthesia, were observed despite the higher plasma concentrations. The Hydro-Oleo technology facilitated the controlled release of β-alanine, ensuring enhanced bioavailability and tolerability. This innovative β-alanine complex demonstrates a safe, effective, and sensory-friendly approach to improving β-alanine absorption, particularly for older adults aiming to maintain good health.

Keywords: β-alanine complex; Hydro-Oleo technology; bioavailability; paresthesia; pharmacokinetics

1. Introduction

Strength, power, and anaerobic threshold are closely linked to muscle quality, especially in older populations (Cadore et al., 2012; Fukumoto et al., 2012). However, age-related muscle loss like sarcopenia leads to declines in these attributes, compromising the ability to withstand fatigue during exercise and daily activities. Additionally, aging is associated with a reduction in muscle buffering capacity and skeletal muscle carnosine levels (Stuerenburg and Kunze, 1999; Tallon et al., 2007), which further increases the risk of fatigue due to a drop in intramuscular pH (Stout et al., 2008). These changes contribute to balance issues, gait instability, and an increased risk of falls in the elderly, ultimately affecting their independence and quality of life (Madureira et al., 2010).

Carnosine, a dipeptide composed of β-alanine and histidine, plays a critical role in buffering hydrogen ions (H^+) in muscle tissue, thereby delaying fatigue during anaerobic activity (Harris et al., 2006; Artioli et al., 2010). Reduced carnosine levels in aging muscle exacerbate acidosis, making routine activities like climbing stairs increasingly challenging. Maintaining carnosine levels is essential for supporting muscle performance, reducing fatigue, and minimizing the risk of falls in older adults (del Favero et al., 2012, Madureira et al., 2010, Stout et al., 2008; McCormack et al., 2013).

Numerous studies have shown that β-alanine supplementation significantly increases muscle carnosine content, enhancing exercise capacity and physical endurance (Baguet et al., 2010, del Favero et al., 2012, Harris et al., 2006, Hill et al., 2007). For instance, del Favero et al. (2012) reported an 85% increase in muscle carnosine levels after 84 days of supplementation in elderly individuals. Similarly, Stout et al. (2008) demonstrated improved physical working capacity following β-alanine supplementation in older adults. Muscle carnosine synthesis is accelerated by the naturally occurring amino acid, β-alanine; which is found in food for example fish and chicken (Harris et al., 2006). When combined with l-histidine, β-alanine enhances the intracellular dipeptide carnosine reserves in muscles and is the amino acid that binds to it at the fastest rate of synthesis. It has an imidazole ring that exerts physiological effects, which is the main mechanism behind its ability to enhance physical performance. (de Salazar et al., 2021). Supplementing with β-alanine rapidly increases plasma β-alanine levels (Harris et al., 2006), which has been demonstrated to significantly increase carnosine content of skeletal muscles (Artioli et al., 2010). The advantages of higher skeletal muscle carnosine content on muscular endurance have been attributed to various mechanisms, such as enhanced

calcium release in type I fibers (Dutka et al., 2012), upgraded antioxidant activity (Boldyrev et al., 2013), enhanced sensitivity of calcium (Dutka et al., 2012) and enhanced skeletal muscle buffering (Sale et al., 2013). Supplementation with β-alanine is accompanied by an enhancement in the capability to execute high-intensity exercise (Harris et al., 2006; Hill et al., 2007; Kendrick et al., 2008, 2009).

Despite these benefits, conventional β-alanine supplementation is often associated with paresthesia, a harmless but unpleasant sensation of tingling or itching caused by rapid absorption and high plasma concentrations (Harris et al., 2006; Décombaz et al., 2012). These effects are attributed to the activation of unmyelinated C-fiber nerve endings and mast cell degranulation, leading to the release of histamine and other pro-inflammatory mediators (Pareja et al., 1998; Brederson et al., 2013). Additionally, β-alanine has been shown to activate transient receptor potential vanilloid 1 (TRPV1) and related sensory receptors, further amplifying the sensory response (Shinohara et al., 2004; Liu et al., 2012). These mechanisms collectively limit the maximum tolerable single dose (Harris et al., 2006; de Salazar et al., 2021) and the feasible daily dosage, thereby prolonging the required supplementation period. Although paresthesia is a harmless sensory side effect, its persistence can deter adherence to high-dosage regimens, which are often necessary for long-term β-alanine supplementation (MacPhee et al., 2013; Crozier et al., 2007). To address these issues, a novel β-alanine complex (TriBsyn™) was developed using Hydro-Oleo technology. This formulation enhances bioavailability while mitigating paresthesia through a controlled-release mechanism.

This study evaluates the pharmacokinetic profile, bioavailability, and tolerability of the β-alanine complex (400 mg TriBsyn™) compared to conventional β-alanine at low (400 mg) and high (1200 mg) doses in healthy aged subjects. Additionally, the symptomatology of paresthesia and β-alanine excretion were assessed to confirm the efficacy of this innovative complex in providing a more user-friendly supplementation option.

2. Materials and methods

2.1. Preparation of β-alanine complex formulation

The innovative formulation, TriBsyn β-alanine complex was prepared using Hydro-Oleo technology comprising a novel β-alanine, β-caryophyllene and caffeine complex and well protected by modified food starch, then coated with taste masking agents to avoid the unpleasant paraesthesia effect as follows: initially, β-alanine-β-caryophyllene complex was prepared by β-alanine dissolved in water containing phospholipids following which β-

caryophyllene is added to the β-alanine mixture and homogenized to form the complexation of β-alanine and β-caryophyllene through the phospholipid bilayer. During the complexation reaction, hydrophobic characteristic β-caryophyllene is linked to hydrophobic part of phospholipids and the hydrophilic amino acid, β-alanine is linked to hydrophilic part of phospholipids, and with the addition of caffeine create a β-alanine-β-caryophyllene complex. The developed β-alanine-β-caryophyllene complex was coated by the addition of aqueous solution of modified food starch with further addition of flavouring agents to mask and remove the unpleasant taste and smell to create the innovative TriBsyn β-alanine formulation complex.

2.2. Characterization of β-alanine complex

A scanning electron microscope (SEM) (Vega3Tescan, Czech Republic) was used to examine the structure and morphology of the β-alanine complex. The sample was scanned at a 5 kV accelerating voltage after being sputter-coated with a small layer of gold by a sputter gold coater and kept in double-sided carbon ribbon-wrapped aluminum stubs. Using DLS-nanoZS, Zetasizer Nanoseries, Malvern Instruments, UK, the dynamic light scattering (DLS) method was used to calculate the zeta potential and mean particle size of the β-alanine complex. The β-alanine complex dispersions were measured at 25 °C after being appropriately diluted with water. The Stokes–Einstein equation was used to calculate the particle sizes. The zeta potential was determined using the Nano DTS software (version 6.34). Each measurement was carried out in a minimum of three sets of 10 runs.

2.3. Stability studies

The stability of β-alanine complex was evaluated under different storage conditions, including 25±2 °C, 40±2 °C with 75±5% RH, and refrigeration at 4±2 °C, all in compliance with ICH guidelines and in a humidity chamber. Glass containers were used to hold the samples during the course of the study. The β-alanine content of the stored samples was measured every 30 days, both before and after storage. All determinations were executed in triplicate. The stability of β-alanine was calculated applying the formula using eq. 1.

$$\textit{Stability of β-alanine (\%)} = 100 \times BA_t/BA_0 \qquad (1)$$

where BA_0 is the initial concentration of β-alanine and BA_t represents the concentration of β-alanine at various sampling points in time.

2.4. Study design

The present study was a randomized, double-blind, single dose, three-treatment, three-way crossover oral bioavailability study of β-alanine complex in comparison with two doses of conventional β-alanine in healthy aged-human adults under fasting conditions. The β-alanine study products (β-alanine complex and conventional β-alanine) were supplied by Natural Alternatives International (NAI), Carlsbad, CA. A total of 12 subjects were enrolled, with a mean age of 52.50 ± 4.52 years, including 7 males and 5 females. who were randomized into three treatment groups. The demographic data, containing height, weight, body mass index (BMI), and vital signs containing systolic blood pressure, diastolic blood pressure, heart rate, and body temperature, were measured and the results are given in Table 1. Twelve subjects were randomized (4+4+4) to receive β-alanine complex (400 mg), conventional β-alanine (400 mg) as low dose and conventional β-alanine (1200 mg) as high dose β-alanine were acutely administered once. A seven-day washout period was included between the crossover events. The primary outcome was pharmacokinetics of β-alanine over an 8-h period and the secondary outcome was to assess the symptoms of Paraesthesia using perceptual ratings questionnaires with Visual Analogue Score (VAS), Qualitative Light Symptoms Inventory (QLSI) and Session-dependent questionnaires after ingestion of each product, which were filled-out during the blood sampling and also estimated the urine excretion of β-alanine.

2.5. Inclusion and exclusion criteria of subjects

2.5.1. Inclusion criteria

The inclusion criteria were normal, healthy, adult, male and female human subjects of age between 50-65 years with a body mass index (BMI) range between 18.50 kg/m^2 to 24.99 kg/m^2. Subject agreed to avoid ß-alanine containing medications and dietary supplements, high activity physical exercise without evidence of underlying disease with normal limits of laboratory values. The subjects were non-smokers and agreed to consume ova-lacto vegetarian diet and agreed to obey to completely necessities of the study protocols.

2.5.2. Exclusion criteria

The exclusion criteria were evidence of allergy or known hypersensitivity to ß-alanine or other related products, subjects with hepatic encephalopathy, cholestasis, myasthenia, pre-existing alcohol abuse, liver disease, liver or renal impairment, existing tinnitus and pre-existing gallbladder disease and any substantial ongoing chronic medical condition or serious illness

that occurred during the last three months. Any illness or condition that may impair any bodily system, including the central nervous system, musculoskeletal, respiratory, gastrointestinal, kidney, liver, cardiovascular, or psychiatric. History of alcohol habit or abuse and malabsorption syndrome that affects β-alanine metabolism. Taking more than 100 mg β-alanine everyday within 2 weeks to screening.

2.6. Ethics and approvals

The study was conducted at Unitree Health Care & Diagnostics, Bengaluru, Karnataka, India, and the study was carried out in accordance with the regulatory and ethical guidelines (Declaration of Helsinki, ICH GCP, and Indian GCP, Schedule-Y). It was accepted and supervised by an ethics committee to safeguard the rights, safety and well-being of all study subjects. Participants were informed of the details of the study prior to signing a consent form. The study protocol was reviewed and approved by ACE Independent Ethics Committee, Bangalore, India on 15 February 2024. The protocol was registered with Clinical Trials Registry India with a registration number: CTRI/2024/03/063457 (clinicaltrials.gov).

2.7. Study procedures

Each subject was administered either β-alanine complex, conventional β-alanine (400 mg) or conventional β-alanine (1200 mg) orally with 240 mL water as determined by the randomization schedule, and a mouth check was directed to confirm compliance. After an overnight fast, the supplements were given orally in a single dose. The product name that the subjects were consuming was unknown to them. The subjects were segregated to prevent any potential influence on their responses on the questionnaires. Four hours into the study, a standardized vegan meal was provided. Between the two crossover periods, a 7-day washout period was maintained.

2.8. Sample collection

A total of 12 (6 mL) pre-dose blood samples were collected, all of which were taken within one hour following the dosage. After dosing, the post-dose samples (6 mL) were taken into vacuum tubes containing K2EDTA at 0.25, 0.50, 1, 2, 4, 6, and 8 h.

2.9. Sample preparation and analytical methods

β-alanine was determined in β-alanine complex and conventional β-alanine formulations in plasma and urine samples by LC-MS analysis used on an Agilent Technologies 1260 infinity

coupled with a Agilent Technologies 6120 Quadrupole mass spectrometer. The sample (0.1 mL) is mixed with 1.9 mL 0.1% formic acid in water, vortexes for 1 minute, and then centrifuged for 5 minutes. The resulting supernatant is efficiently filtered through a 0.22 µm syringe filter. The resultant sample is directly injected into the LC-MS system equipped with a reversed-phase column and set to a mobile phase of 0.1% formic acid in acetonitrile and 0.1% formic acid in water with the following conditions of HPLC systems: Agilent Eclipse plus C-18 column (150mm x 4.5 mm × 3.1µm) heated to 35 °C using isocratic method with mobile phase A (0.1 % formic acid in water): mobile phase B (0.1% formic acid in acetonitrile) as 60:40 v/v ratio at a flow rate of 0.8 mL/min with a run time of 10 min. The mass spectrometer was used in positive ion mode to get the mass spectra of β-alanine when it was connected to an electrospray ion source.

2.10. Paraesthesia Questionnaires

Paraesthesia was measured concurrently after each blood sample collection using perceptual ratings questionnaires with Visual Analogue Score (VAS), Qualitative Light Symptoms Inventory (QLSI) and Session-dependent questionnaires.

2.10.1. Visual Analogue Score (VAS)

The perceived severity of symptoms was scored using Visual Analogue Score (VAS) (Décombaz et al., 2012). It consists of a horizontal, continuous 9-cm line with vertical marks 2 mm from each end, labelled ‘‘no unusual sensation’’ to ‘‘most intense sensation imaginable’’. A box is placed next to the low end where subjects acknowledge with a tick their awareness of the direction of the scale. The record is a vertical line drawn at the level most fitting with perception intensity. VAS is expressed as percent of scale length from the low end.

2.10.2. Qualitative Light Symptoms Inventory (QLSI)

The Qualitative Light Symptoms Inventory (QLSI) qualified the nature of the sensation from within a subset of six descriptive attributes: ‘‘pins and needles and/or tingles’’, ‘‘tickling and/or itching’’, ‘‘flush and/or shiver’’, ‘‘tactile hypersensitivity and/or irritation’’, ‘‘numbness and/or insensitivity’’, and ‘‘pain and/or soreness’’. Next to each attribute is a 5-level scale from 0 = ‘‘absent’’ to 4 = ‘‘extremely intense’’. Subjects were recorded the most fitting level for each descriptor (Décombaz et al., 2012).

2.10.3. Session-dependent questionnaires

Three questionnaires were filled retrospectively at the end of the test, including Q1: the Profile of Mood States (POMS), a French adaptation of the abridged profile of mood (Fillion and

Gagnon 1999), Q2: the Questionnaire de Douleur de Saint-Antoine (QDSA), a validated French version of the McGill Pain Questionnaire (Boureau et al. 1992) that describes sensory and emotional dimensions of pain, and Q3: the Spielberger State Anxiety Inventory (Spielberger et al. 1983): Mood and anxiety were monitored to account for the potential input of affective states on sensory scores (Villemure and Bushnell 2002).

2.11. Statistical analysis

Pharmacokinetic parameters including C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ for all three investigational product groups were created and stated as the mean±SD. Statistical analysis was executed utilizing SPSS statistical software version 16, the "p" value < 0.05 was considered as a significant difference. The paired t-test and ANOVA were used to measure the change between the study groups.

3. Results and discussion

In this study β-alanine complex was successfully designed and developed by Hydro-Oleo technology, which was characterized by different instrumental techniques such as SEM, particle size distribution and zeta potential measurements. The study also involved a randomized, double-blind, single dose, three-treatment, three-way crossover design to assess oral bioavailability of β-alanine complex in comparison with two different doses of conventional β-alanine in 12 healthy aged-human adults under fasting conditions. The participants using any of the three formulations reported no side effects. The effects of a novel β-alanine complex were compared with conventional β-alanine (400 mg) and conventional β-alanine (1200 mg) formulation.

3.1. Characterization of β-alanine complex

3.1.1. Scanning electron microscope (SEM)

The morphological appearance of the β-alanine complex was examined by SEM, as shown in Fig. 2. The β-alanine complex is present as uniformly sized nanoparticles with spherical forms and smooth surfaces, free of any aggregation, indicating the stability of the formulation because of the cutting-edge Hydro-Oleo technique that produced the complex.

3.1.2. Particle size distribution and zeta potential measurement

One of the most crucial aspects of nutraceutical formulations that affects their solubility, release rate, physical stability, chemical stability, and biological performance is particle size (Tamjidi

et al., 2013). The particle size of the β-alanine complex was obtained in the range of 109.4 to 237.5 nm with an average particle size of 171.3±4.6 nm as shown in Fig. 3(a), which clearly revealed that the sizes of the particles exist in a narrow range, and are uniform. This indicated the β-alanine complex exists in homogeneous size distribution.

The physical characteristics of drug delivery systems are examined using zeta potential. It indicates both the stability and surface electrical charges of drug delivery systems; the more stable the drug delivery system, the higher the potential since it increases particle repulsion. A high zeta-potential (> ±30 mV) is beneficial to formulations physical stability as it prevents aggregation between formulated particles due to electrostatic repulsion (Müller et al., 2001). Small zeta potential values lead to flocculation and particle aggregation because of the van der Waals force of attraction between the particles, which makes the formulations physically unstable. The β-alanine complex exhibited a negative surface charge with a higher value of -31.2 mV than -30 mV (Fig. 3(b)). This high negative zeta potential obtained for this β-alanine complex accounts for strong electrostatic repulsion between the particles, which prevents vesicles aggregation and fusion, which also supports the homogeneous size distribution of the particle size data Fig 3(a)) and SEM results (Fig. 2).

3.2. Stability studies

Storage stability of the complex formulations plays a crucial role in determining whether or not the food, beverage, and nutraceutical industries can use it. The stored β-alanine complex formulations did not undergo any changes in physical appearance or color over the 180-day study period at 4 °C, 25 °C, and 40 °C. Table 2 summarizes the amount of β-alanine present in the stability measurements under various conditions. The findings indicated that, after 180 days of storage at all investigated temperatures (4 °C, 25 °C, and 40 °C), there is no discernible change in the β-alanine content, indicating that the β-alanine complex is quite stable.

3.3. Pharmacokinetic analysis

Table 3 provides the total average pharmacokinetic characteristics (mean ± SD) for each formulation derived from plasma β-alanine along with the p-values. Figure 4 shows the graphical representations of the mean concentrations of three formulations as a function of time.

Area under the plasma concentration versus time curve ($AUC_{0\text{-}8h}$) as determined by the linear trapezoidal method from time zero to the last measurable concentration and the results are given in Table 3. $AUC_{0\text{-}8h}$ was 601.75 µmol.h/L, 135.11 µmol.h/L and and 470.25 µmol.h/L for

β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg), respectively. Based on the AUC_{0-t} values, the bioavailability of β-alanine complex (400 mg) is significantly higher ($P<0.001$) than the two different doses of conventional β-alanine (400 mg) and conventional β-alanine (1200 mg). The extent of absorption of the β-alanine complex based on the AUC_{0-t} was 4.45 and 1.28-folds greater than the low dose conventional β-alanine and high dose conventional β-alanine formulations respectively. Furthermore, high dose conventional β-alanine has 3.48-fold higher absorption than the low dose conventional β-alanine formulation, whereas low dose of β-alanine complex has 4.45-fold higher absorption, that clearly revealed the higher bioavailability of the β-alanine complex formulation.

Maximum measured plasma concentration of β-alanine (C_{max}) was 123.09±10.83 µmol/L, 41.91±3.11 µmol/L and 148.79±10.44 µmol/L for β-alanine complex (400 mg), conventional β-alanine (400 mg) and conventional β-alanine (1200 mg) respectively (Table 3 and Fig. 4). Based on the overall C_{max} values, the bioavailability of β-alanine complex (400 mg) is significantly higher ($P<0.01$) than the low dose conventional β-alanine (400 mg) and slightly lower than the conventional β-alanine (1200 mg).

The time of the maximum measured plasma concentration (T_{max}) was 2 hours for all three groups (Table 3) yet the T_{max} obtained in the same time after oral administration of β-alanine complex suggests more efficient β-alanine absorption and bioavailability due to the Hydro-Oleo technology complexation of β-alanine in the formulation.

Elimination rate constant (K_{el}) is calculated using the formula (eq. 2) by two different time points after elimination of the study products.

$$k_e = CL/Vd = \ln(C_1/C_2)/(t_2-t_1) \qquad \textbf{(2)}$$

K_{el} was 0.29, 0.40 and 0.66 for β-alanine complex (400 mg), conventional β-alanine (400 mg) and conventional β-alanine (1200 mg), respectively (Table 3). The lower K_{el} value for the β-alanine complex explains the lower elimination of β-alanine because of layered release and different absorption pathways of β-alanine from the β-alanine complex formulation due to the well-complexation by the Hydro-Oleo technology of β-alanine in the formulation.

The elimination or terminal half-life calculated as $0.693/ K_{el}$. $t_{½}$ was 2.36 h, 1.72 h and 1.06 h for β-alanine complex (400 mg), conventional β-alanine (400 mg) and conventional β-alanine (1200 mg), respectively (Table 3). The higher $t_{½}$ value for the β-alanine complex explains the

longer availability of β-alanine from the β-alanine complex formulation due to the well-complexation by the Hydro-Oleo technology of β-alanine in the formulation.

The area under the plasma concentration versus time curve from time zero to infinity ($AUC_{0-\infty}$) is calculated using eq. 3. and the results are given in Table 3.

$$AUC_{0-\infty} = AUC_{0-t} + C_t / K_{el} \longrightarrow \textbf{(3)}$$

where C_t is last measurable concentration and K_{el} is terminal elimination rate constant

$AUC_{0-\infty}$ was 681.50 µmol.h/mL, 144.41 µmol.h/mL and 478.32 µmol.h/mL for β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg), respectively. Based on the $AUC_{0-\infty}$ values, the bioavailability of β-alanine complex (400 mg) is significantly higher ($P<0.001$) than the two different doses of conventional β-alanine (400 mg) and conventional β-alanine (1200 mg). The extent of absorption of the β-alanine complex based on the $AUC_{0-\infty}$ was 4.72 and 1.43-folds increase over the low dose conventional β-alanine and high dose conventional β-alanine formulations respectively. Furthermore, high dose conventional β-alanine has 3.31-fold higher absorption than the low dose conventional β-alanine formulation, whereas low dose of β-alanine complex has 4.72-fold higher absorption, which clearly indicated the higher bioavailability of the β-alanine complex formulation.

Supplementing with β-alanine improved exercise tolerance and reversed the executive function deficits brought on by endurance exercise. The elderly population can also continue to benefit from exercise with increased safety by addressing the executive function impairments that come with endurance exercise. (Furst et al., 2018). Age-related decreases in skeletal muscle carnosine have been connected to a decrease in muscle buffering capacity and, theoretically, an increase in the rate at which fatigue occurs during exercise. A 90-day study on the supplementation of β-alanine in the elderly showed significant improvements in muscle endurance and physical working capacity at the fatigue threshold. This was attributed to improved intracellular pH control, which was caused by a greater capacity to buffer H^+. This could potentially support the maintenance of health and independent living in older men and women (Stout et al., 2008). Accordingly, the highly bioavailable form of β-alanine complex can be utilized to the elderly population to improve their muscle endurance and physical working capacity.

3.4. Excretion of β-alanine

Excretion of β-alanine through urine is also analysed in all the study groups, which were shown, there is no significant detection of β-alanine. However, less than 5% of β-alanine excretion without modifications has been reported in earlier investigations (Harris et al., 2006; Décombaz et al., 2012; Stegen et al., 2013; Stautemas et al., 2020). Even so, a small amount lost in urine

would not translate into a larger uptake by target cells since, as previously demonstrated, it might be removed by metabolic pathways (Pihl et al., 1955; Stegen et al., 2013; Stautemas et al., 2020).

3.5. Paraesthesia Questionnaires

3.5.1. Visual Analogue Score (VAS)

The sensory side-effects based on the VAS is depicted in the Fig. 5. The VAS score of 0.62 was significantly lower ($P<0.001$) in the β-alanine complex group than both conventional β-alanine groups, which scored 2.02 and 4.01, for conventional β-alanine (400 mg) and conventional β-alanine (1200 mg) respectively. In the β-alanine complex group most subjects did not exceed the VAS score of 1/10 (0.62). Both the conventional β-alanine formulations registered a higher VAS score, 2/10 and 4/10 for low dose conventional β-alanine and high dose conventional β-alanine, respectively. Moreover, the high dose β-alanine formulation exceeded the VAS by more than 3 and produced the symptoms of paraesthesia. The results demonstrate that the β-alanine complex registered higher bioavailability but did not cause paraesthesia due to the Hydro-Oleo complexation technology.

3.5.2. Qualitative Light Symptoms Inventory (QLSI)

The sensory side-effects based on the Qualitative Light Symptoms Inventory (QLSI) questionnaire for intensity of the symptoms identified as pins-and-needles, tingle; tickling, itching; flush, shiver; tactile hypersensitivity, irritation; numbness, insensitivity; and pain, soreness is depicted in the Fig. 6. All the quantified sensation parameters for β-alanine complex are significantly lower ($P<0.001$) than both conventional β-alanine formulations. Moreover, the sensation parameters for β-alanine complex registered almost “absent” category, whereas the conventional β-alanine (400 mg) group registered “mild to moderate” sensation and the conventional β-alanine (1200 mg) group registered “moderate to strong”. The results demonstrated conventional β-alanine produced unusual sensations.

In an earlier study, Harris et al. (2006) reported "mild symptoms of flushing" in two of the four subjects using 800 mg dosages (10 $mg.kg^{-1}$), but in another study, Hill et al. (2007) reported "infrequent and mild when they occurred." According to Sweeney et al. (2010), there were "no side effects other than a mild prickling sensation" in the limbs and neck. An apparent plasma threshold is crossed, and side effects are regarded as "unpleasant" at single β-alanine dosages of 3.2 g (40 $mg.kg^{-1}$) and above (Harris et al. 2006). In this study, high dose conventional β-alanine (1200 mg) groups registered moderate to strong QLSI scores for all sensation

parameters because of the high plasma concentration of β-alanine, whereas β-alanine complex (400 mg) did not register QLSI sensation parameters. Even though β-alanine complex (400 mg) has a high plasma concentration, the absence of paraesthesia could likely be due to the presence of β-caryophyllene, known to have anti-inflammatory action. Studies have shown oral β-caryophyllene administration decreased spinal neuroinflammation, weakened mechanical allodynia and thermal hyperalgesia, and lowered inflammatory pain responses. β-caryophyllene also inhibited inflammation and tissue damage in model of colitis and nephrotoxicity (Klauke et al., 2014; Bento et al., 2011; Horvath et al., 2012).

3.5.3. Session-dependent questionnaires

3.5.3.1. Profile of Mood States (POMS)

The Profile of Mood States (POMS) is a commonly used tool for evaluating someone's mood. It is therefore extremely pertinent to a wide range of clinical and social psychology research concerns. In this study, all the quantified mood related parameters for the β-alanine complex group have a significantly positive impact ($P<0.001$) on the mood when compared with both conventional β-alanine groups (Fig. 7). Aging is linked to higher levels of oxidative stress, neuroinflammation, and mood disorders, all of which can affect cognitive performance. A study found that supplementing with β-alanine enhances fractional anisotropy scores in the hippocampus and amygdala of men and women aged 60–80 years, while having no effect on brain-derived neurotrophic factor or inflammatory markers. Nonetheless, notable advancements in fractional anisotropy values were observed in the right hemisphere's hippocampal region and the left hemisphere's amygdala, together with a tendency towards an increased total volume inside the β-alanine group (Ostfeld et al. 2024). A second study also indicated that supplementing with β-alanine improved the cognitive performance of elderly persons whose baseline cognitive capabilities were below normal to borderline. Additionally, the study demonstrated that the supplementing with β-alanine was able to lessen depressive symptoms (Ostfeld et al., 2023). The results of POMS in the β-alanine complex show significant effects on mood, neuroprotection, antioxidant effect and cognitive function.

3.5.3.2. Questionnaire de Douleur de Saint-Antoine (QDSA)

Questionnaire de Douleur de Saint-Antoine (QDSA), a validated French version of the McGill Pain Questionnaire (Boureau et al. 1992) that describes sensory and emotional dimensions of pain. Any painful aspects of the feelings experienced throughout the study were assessed retrospectively using the QDSA questionnaire. In the QDSA questionnaire, the minimum pain

score is 0, which would not be seen in a person with true pain, the maximum pain score is 78, the higher the pain score, the greater the pain. The β-alanine complex group registered very minimum QDSA score (3.33) indicating there were no pain related issues in the β-alanine complex group (Fig. 8). Furthermore, the QDSA score was significantly lower ($P<0.001$) in the β-alanine complex group (3.33) than the both conventional β-alanine groups, which are registered 19.92 and 33.33, for conventional β-alanine (400 mg) and conventional β-alanine (1200 mg) respectively. Overall, all three groups registered low on the pain scale.

The pain intensity was also described using five different words such as mild, discomforting, distressing, horrible and excruciating in all the study group subjects and the results are given in Fig. 8. The β-alanine complex group registered very mild pain intensity, whereas the conventional β-alanine (400 mg) group registered mild to discomforting, likewise the conventional β-alanine group (1200 mg) registered between discomforting and distressing, which reveals that the β-alanine complex did not show any sensory and emotional dimensions of pain.

The β-alanine complex group registered a very minimum pain intensity score, which indicates there is no pain related issues in the β-alanine complex group (Fig. 9). Furthermore, the pain intensity score was significantly lower ($P<0.001$) in the β-alanine complex group than the both conventional β-alanine groups, which are registered nearly 2 and 3, for conventional β-alanine (400 mg) and conventional β-alanine (1200 mg) respectively, indicating the condition of discomfort and distress.

Without any intervention, older age people continue to see a decline in working capacity, muscle mass, and muscle function (Marcus et al., 2012). β-alanine supplements improved working capacity of older adults without a training intervention (del Favero et al., 2012; Stout et al., 2008). Supplementing with β-alanine may lead to improvements in working capacity and perhaps encourage older adults to maintain a more active lifestyle. Earlier studies showed that, the high dose of β-alanine produced paraesthesia as well as high pins and needles intensity and which are correlated with high C_{max} and AUC values (Harris et al., 2006; Décombaz et al., 2012; de Salazar et al., 2021). In this study, even though β-alanine complex registered high C_{max} and AUC values, it did not demonstrate paraesthesia or other typical effects attributed to β-alanine due to the presence of β-caryophyllene, a Phytocannabinoid, in this β-alanine complex formulation.

The endocannabinoid system has been shown to have a significant role and function as a primary healing target in the modulation of paresthesia and pain (Anand et al., 2010; Ligresti et al., 2014). β-caryophyllene is the most prominent naturally occurring cannabinoid receptor type 2 (CB2) receptor and a powerful phytocannabinoid with anti-inflammatory effects, because it performs an essential role in pain variation, which may hold promise for paresthesia and pain management. β-caryophyllene functions as a selective binder for the CB2 receptor, which is the therapeutic target for the treatment of paresthesia, osteoporosis, inflammation, pain, and atherosclerosis, among other conditions.

3.5.4. State Trait Anxiety Inventory (STAI)

The State Trait Anxiety Inventory (STAI) is a validated 20 item self-report assessment device which includes separate measures of state and trait anxiety. STAI is an appropriate and adequate measure for studying anxiety in research and clinical settings. The results of STAI questionnaire clearly reveals that the β-alanine complex group did not report any anxiety related issues, whereas, both the conventional β-alanine group subjects registered minimal anxiety related issues (Fig. 10). In this study, the β-alanine complex group had a significantly positive impact ($P<0.001$) on all the quantified anxiety related parameters when compared with both conventional β-alanine groups (Fig. 10).

Overall, the study demonstrates that β-alanine complex increases the absorption of β-alanine when compared to both conventional β-alanine supplementations. The 12 subjects enrolled were each administered β-alanine complex formulation, and conventional β-alanine with sufficient washout period to determine oral bioavailability of β-alanine delivered as β-alanine complex (400 mg), conventional β-alanine (400 mg) and conventional β-alanine (1200 mg)) in a randomized, double blind, single dose, three treatments, three-way crossover in healthy, adult, aged human participants under fasting conditions. In the study period, 8 blood samples were collected including the pre-dose sampling in each period. For the pre-dose blood sample (00.00 hours) 5 ml was collected within 60 minutes prior to dosing. Post dose blood samples 5 ml was collected at 15, 30, 60, 120, 240, 360 and 480 minutes.

The maximum β-alanine concentration for the β-alanine complex (400 mg) group was registered as 123.09 ± 10.83 µmol/L, which was significantly higher ($p<0.01$) than that for the conventional β-alanine (400 mg) as 41.91 ± 3.11 µmol/L and almost similar concentration with the high dose conventional β-alanine (1200 mg) as 148.79 ± 10.44 µmol/L. The extent of absorption of β-alanine for the β-alanine complex was a 4.45-fold increase over the

conventional β-alanine (400 mg) formulation and 1.28-fold increase over the conventional β-alanine (1200 mg) formulation, thus confirming an increased bioavailability. Furthermore, a longer half-life period (2.36 h) was registered for the β-alanine complex than the conventional β-alanine (400 mg) and the conventional β-alanine (1200 mg) formulations at 1.72 and 1.06 h respectively, which can be attributed to the ability of the Hydro-Oleo encapsulation technology to gradually release the encapsulated β-alanine.

Paraesthesia measurements were performed through VAS and QLSI perceptual rating scales as well as session-dependent QDSA and STAI questionnaires. β-alanine complex registered significantly lower VAS and QDSA scores and lower or absent sensations such as pins, needles, tingles, itching, numbness, soreness, pain, etc. without the effects of paraesthesia or any other side effects. Furthermore, the β-alanine complex registered a significantly positive impact on mood and anxiety.

4. Conclusion

In summary, this study demonstrates that oral delivery of the β-alanine complex (400 mg) produced circulating concentrations of β-alanine nearly 4.5-fold and 1.3-fold increase over those elicited by oral delivery of conventional β-alanine (400 mg) and conventional β-alanine (1200 mg) respectively due to the well-complexation of β-alanine by the Hydro-Oleo technology. The increased plasma concentration of β-alanine was not associated with any adverse effects over the study period, suggesting that this β-alanine complex provides a suitable means to enhance β-alanine bioavailability. Even though β-alanine complex (400 mg) achieved high plasma concentrations it did not produce paraesthesia. This could be due to the inclusion of the phytocannabinoid β-caryophyllene in the β-alanine Hydro Oleo complex providing anti-inflammatory effect via selective binding of the CB2 receptor, a therapeutic target for management of paraesthesia. Adequate availability of β-alanine is critical for maintaining health, protecting the body from toxins, and promoting longevity. The apparent good safety and sensory profile of the β-alanine complex suggests that the β-alanine complex formulation can be used as a supplement to maintain good health particularly in aged people.

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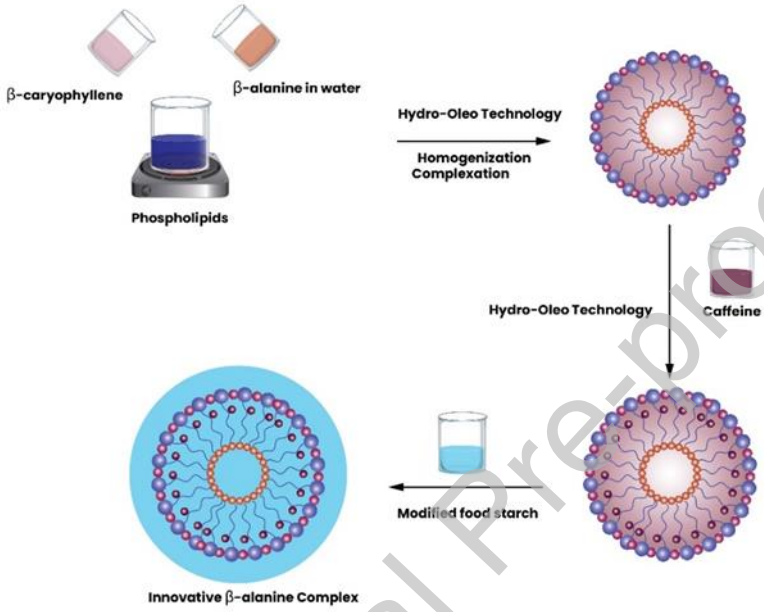


Fig. 1. Schematic representation of the preparation method of β-alanine complex formulation by Hydro-Oleo technology

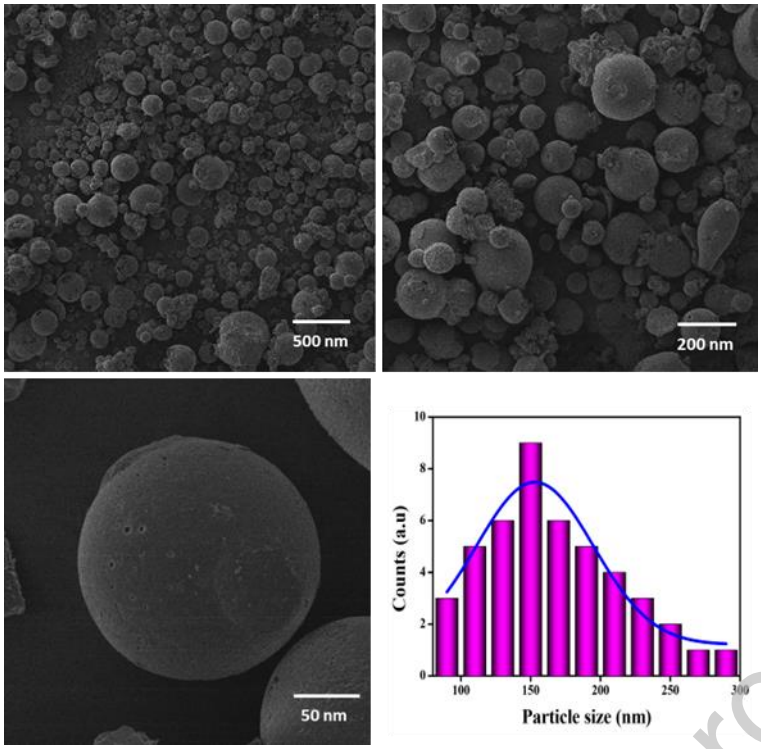


Fig. 2. SEM photographs with different magnifications and histogram image of the β-alanine complex

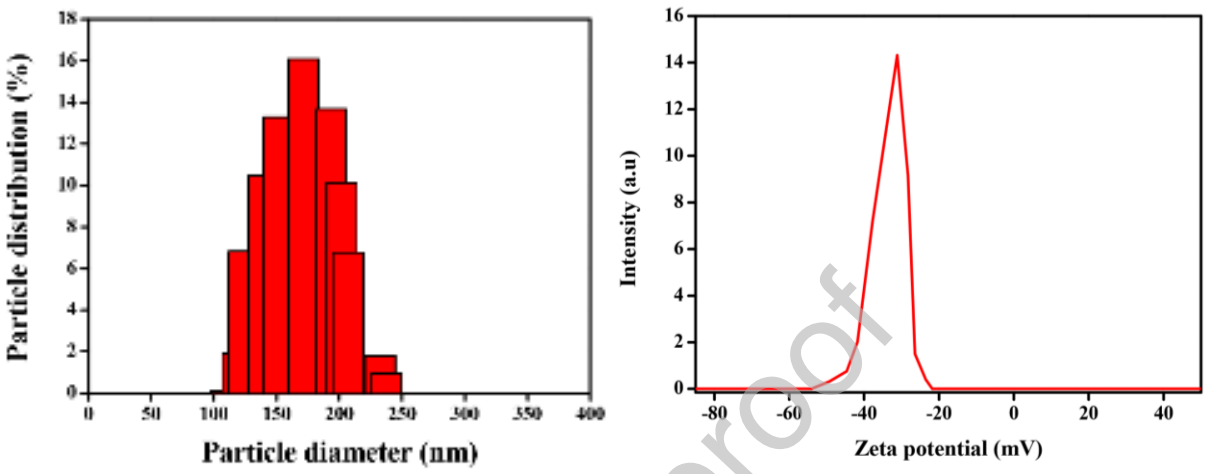


Fig. 3. Particle size distribution (a) and Zeta potential measurement (b) of the β-alanine complex

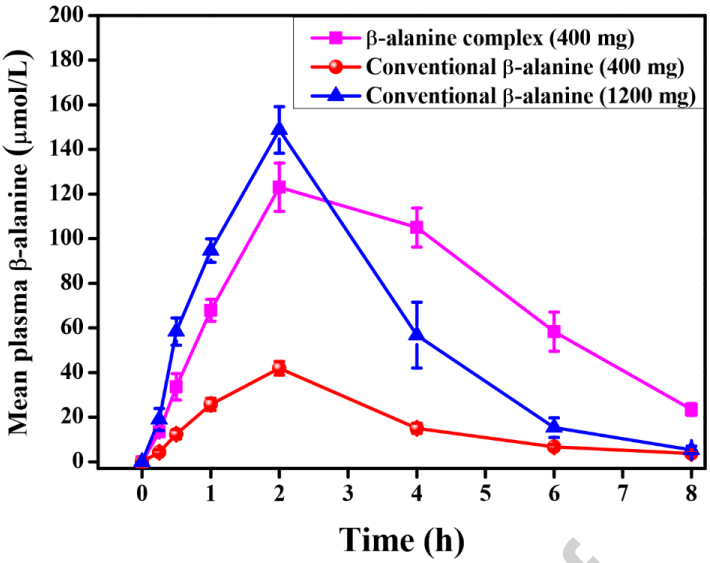


Fig. 4. Mean plasma β-alanine concentrations (μmol/L) of β-alanine complex (400 mg) compared with conventional β-alanine (400 mg) and conventional β-alanine (1200 mg). All of the values stated are mean ± SD.

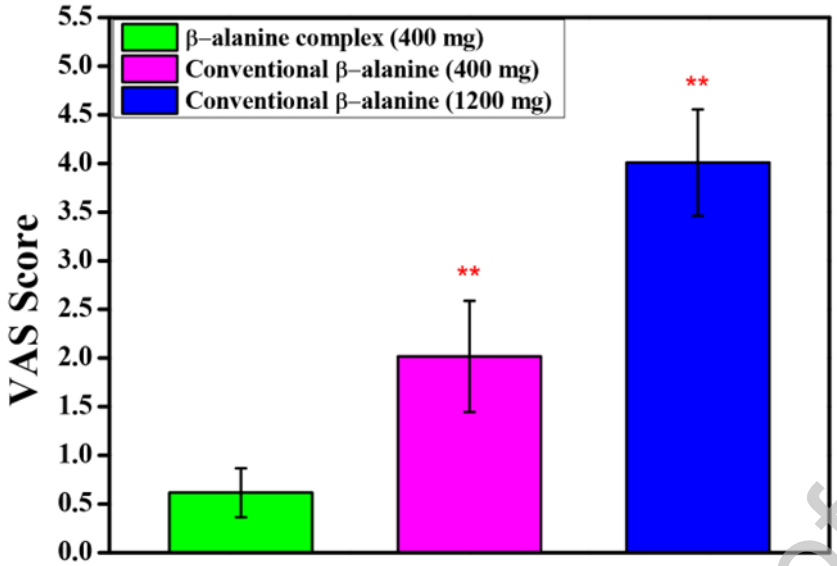


Fig. 5. Visual Analogue Score (VAS) for β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg)

Paired t-test used for comparisons with β-alanine complex (400 mg) at $P < 0.05$ level; **P<0.001.

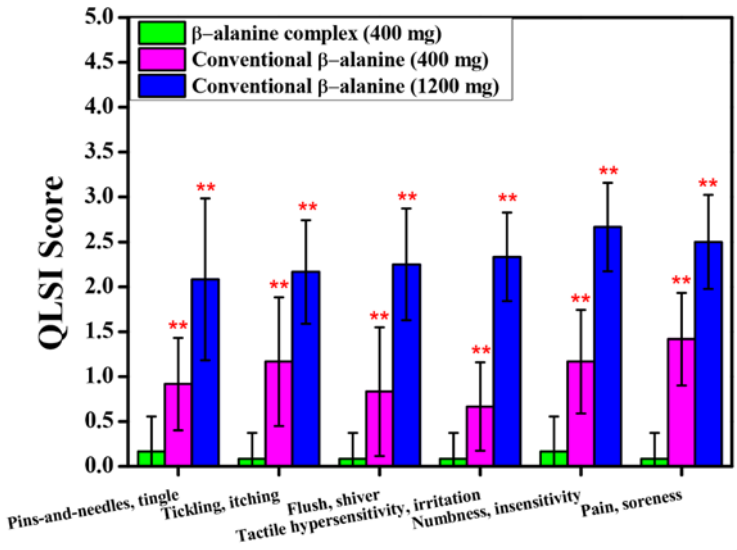


Fig. 6. Qualitative Light Symptoms Inventory (QLSI) Questionnaire for β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg). Paired t-test used for comparisons with β-alanine complex (400 mg) at $P < 0.05$ level; **P<0.001

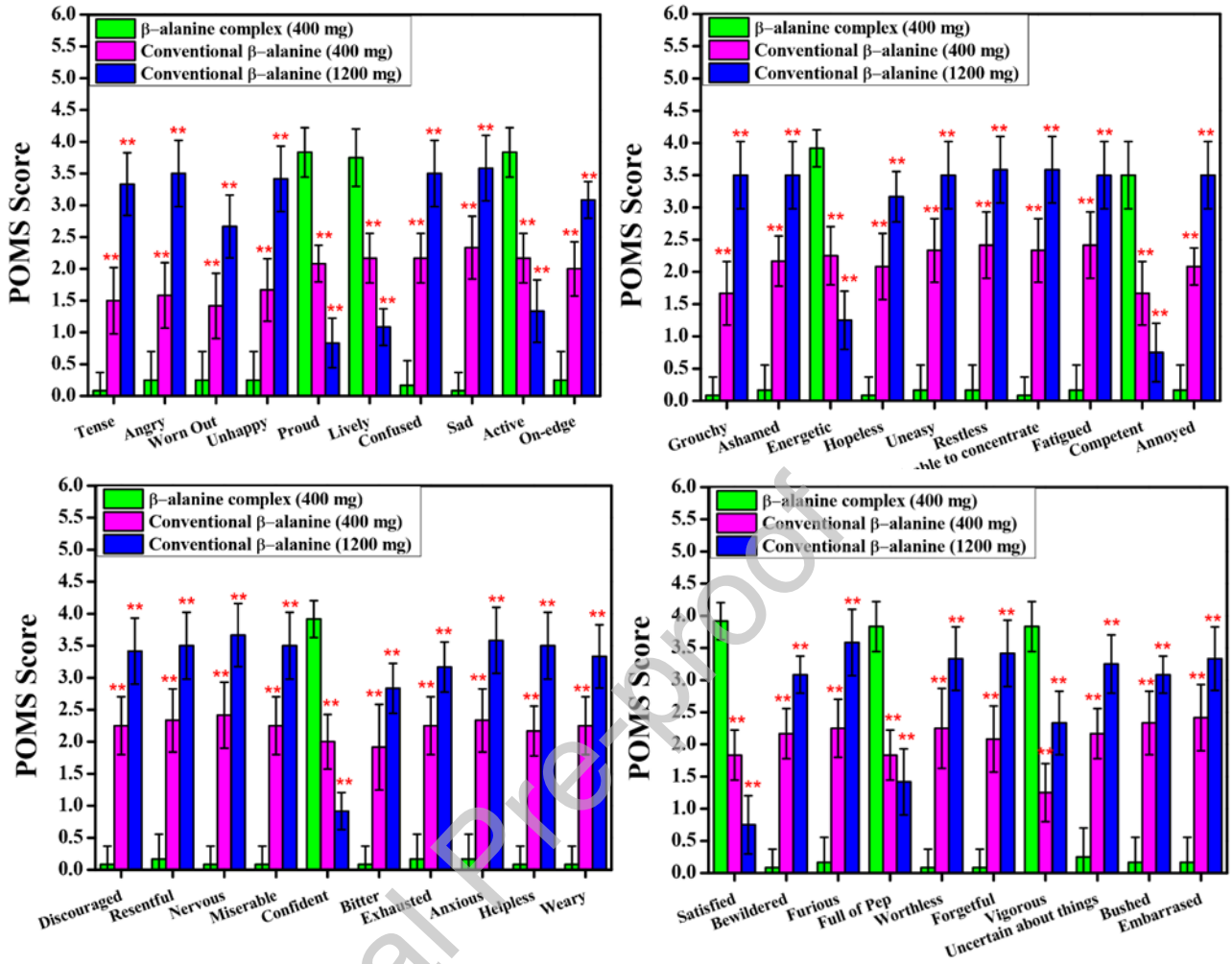


Fig. 7. Profile of Mood States (POMS) Questionnaire for β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg).

Paired t-test used for comparisons with β-alanine complex (400 mg) at $P < 0.05$ level; **P<0.001

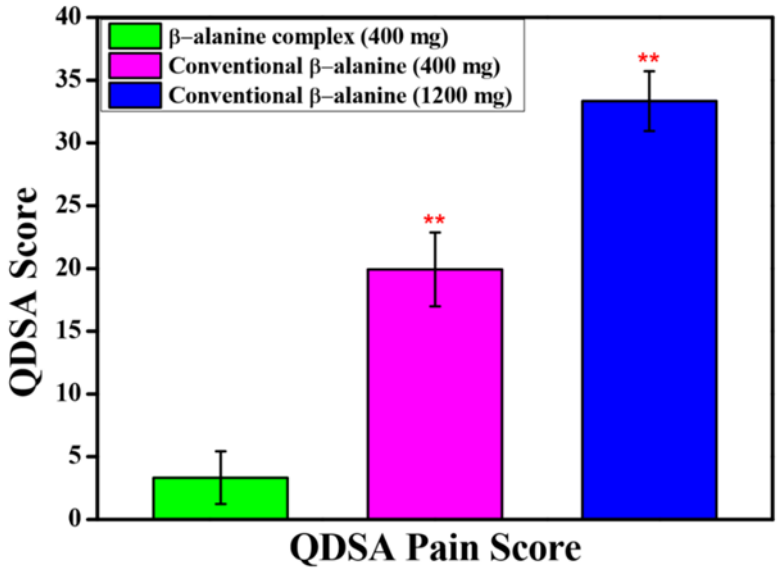


Fig. 8. Questionnaire de Douleur de Saint-Antoine (QDSA) for β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg). Paired t-test used for comparisons with β-alanine complex (400 mg) at $P < 0.05$ level; **$P<0.001$

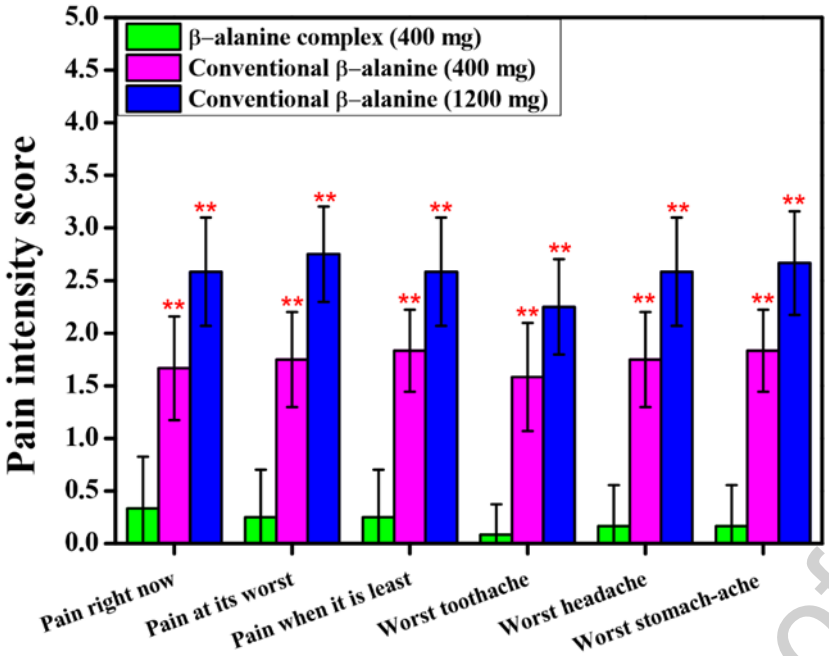


Fig. 9. Pain intensity based on the QDSA Questionnaire for β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg). Paired t-test used for comparisons with β-alanine complex (400 mg) at $P < 0.05$ level; **P<0.001

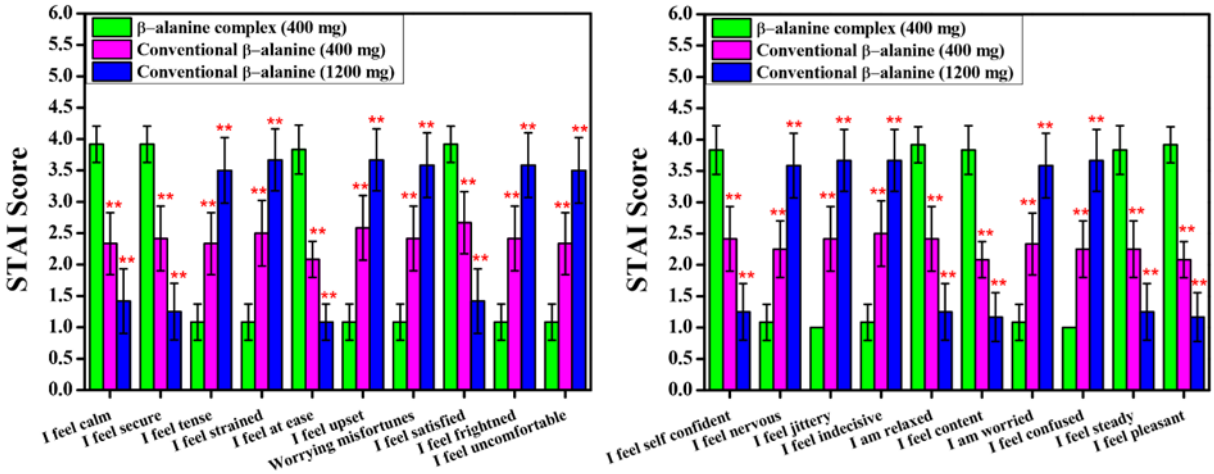


Fig. 10. State Trait Anxiety Inventory (STAI) Questionnaire for β-alanine complex (400 mg), Conventional β-alanine (400 mg) and Conventional β-alanine (1200 mg). Paired t-test used for comparisons with β-alanine complex (400 mg) at $P < 0.05$ level; **P<0.001

Table 1. Subject demographics (N=12; Male 7 and Female 5)

Demographics	Mean ± SD	Range (Min, Max)
Age (Years)	52.50 ± 4.52	48, 64
Height (m)	164.69 ± 8.59	153.4, 173.4
Weight (Kg)	58.11 ± 2.98	53.9, 62.2
Body mass index (Kg/m^2)	21.49 ± 1.53	18.9, 23.7
Systolic blood pressure (mm Hg)	124.33 ± 3.70	118, 130
Diastolic blood pressure (mm Hg)	72.50 ± 3.21	68, 78
Heart rate (bpm)	75.00 ± 2.63	70, 78
Body temperature (ºF)	95.63 ± 0.40	95.3, 96.7

Table 2. β-alanine content in the β-alanine complex under different stability conditions

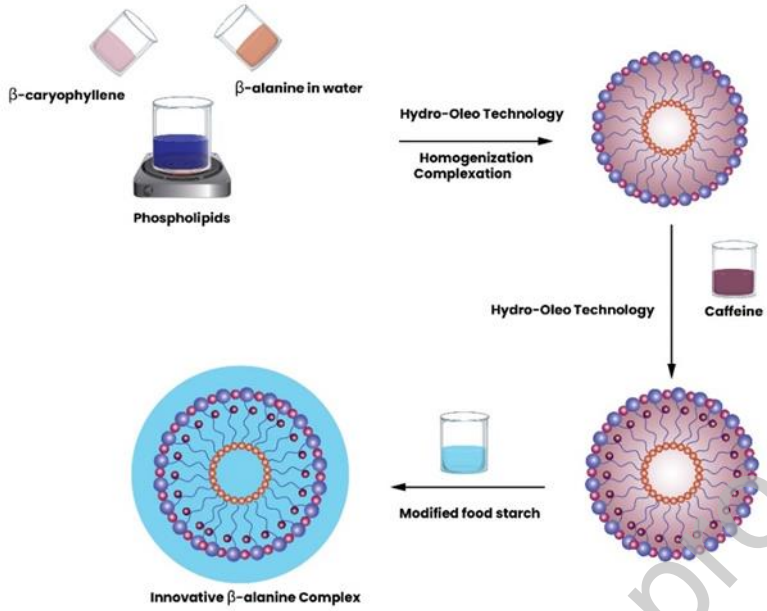
Stability condition	β-alanine content (%)						
	Initial	30 days	60 days	90 days	120 days	150 days	180 days
4 ± 2 °C	99.62 ± 0.27	99.18 ± 0.22	99.01 ± 0.21	98.94 ± 0.18	98.88 ± 0.26	98.79 ± 0.24	98.71 ± 0.19
25 ± 2 °C	99.62 ± 0.27	99.07 ± 0.31	98.89 ± 0.14	98.72 ± 0.31	98.65 ± 0.23	98.45 ± 0.17	98.36 ± 0.24
40 ± 2 °C	99.62 ± 0.27	98.82 ± 0.36	98.46 ± 0.32	98.32 ± 0.31	98.14 ± 0.42	98.09 ± 0.35	98.04 ± 0.37

Table 3. The average PK variables (mean ± SD) from plasma β-alanine of β-alanine complex (400 mg) compared with conventional β-alanine (400 mg) and conventional β-alanine (1200 mg) and the p-value

PK parameters	β-alanine complex (400 mg)	Conventional β-alanine (400 mg)	Conventional β-alanine (1200 mg)	P values
C_{max} (µmol/L)	123.09±10.83	41.91±3.11	148.79±10.44	<0.01*
Fold – Compared with Conventional β-alanine (400 mg)	**2.94**		**3.55**	
AUC_{0-t}=AUC_{0-8h} (µmol.h/L)	601.75	135.11	470.25	<0.001**
Fold – Compared with Conventional β-alanine (400 mg)	**4.45**		**3.48**	
T_{max} (h)	2.00	2.00	2.00	-
$t_{½}$ (h)	2.36	1.72	1.06	
K_{el}	0.29	0.40	0.66	
$AUC_{0-\infty}$ (µmol.h/mL)	681.50	144.41	478.32	<0.001**
Fold – Compared with Conventional β-alanine (400 mg)	4.72		**3.31**	

Comparison between the study groups using ANOVA with the level of significance (α) as p<0.05; Statistically significant at *P<0.01 and **P<0.001

Graphical Abstract



Author Statement

Sreeraj Gopi: Conceptualization, Methodology, Validation, Writing-Original Draft **Augustine Amalraj:** Supervision, Methodology, Software, Investigation, Writing – Review and Editing **Di Tan** Methodolog, Validation, Resources, Writing- Review and Editing

Ethical Statement

Participants were informed of the details of the study prior to signing a consent form. The study protocol was reviewed and approved by ACE Independent Ethics Committee, Bangalore, India on 15 February 2024. The protocol was registered with Clinical Trials Registry India with a registration number: CTRI/2024/03/063457 (clinicaltrials.gov).

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for *[Journal name]* and was not involved in the editorial review or the decision to publish this article.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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