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Evaluation of chemical composition, anti-oxidant and anti-inflammatory potential of herbs enriched plant-based protein mix for women athletes

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Abstract
Ergogenic aids enable an athlete to adapt to their specific sport training and improve the effectiveness of exercise, and boost recovery from exercise. Heavy and sustained intensity exercises are known to increase pro-inflammatory markers causing decreased performance and recovery in an athlete. From the scientific literature survey we also observed that dietary intake of female athletes is inadequate and many of them suffer macro and mineral deficiencies. Supplements may be necessary to fill any nutritional gaps and therefore the present study was targeted towards developing a cost-effective plant-based ergogenic aid for women athletes. Plant based sources high in essential amino acids and bioactive components having performance enhancing, antioxidant, immune enhancing, pain relieving, and fatigue reducing properties are potential candidates that can be explored for optimizing the performance of athletes. In the present study, we standardized and formulated a performance enhancing plant-based instant mix. The most acceptable variant was evaluated for its chemical composition and *in vitro* antioxidant and anti-inflammatory potential. The developed mix showed comparable levels of crude protein (28±0 gm/100g), carbohydrate (56±0.03 gm/100 gm) and calcium (32±0.07 mg/100g) with commercial protein mix. Significantly higher levels were observed for phytochemicals (mg/100g) *viz*., polyphenols (597±31.8) and flavonoids (226±0.05). The methanolic extract of the instant mix also exhibited significant ($p<0.005$) scavenging action of DPPH radicals (84.13±0.02), FRAP (3.39±0.02), reducing power (0.89±0.01), anti-hyaluronidase activity (39.4±0.02) and anti-protease activity (93.68±0.13) at 20µg/ml concentration, making them great supplements for muscle building, muscle maintenance and thereby optimal performance as exactly required by athletes.

Keywords: Ergogenic aid, plant-based protein, anti-oxidant, anti-inflammatory, women athletes

Introduction
Ergogenic aids are substances or devices that enable an athlete to adapt to their specific sport trainings and improve workout performance capability. This includes aids that help prepare an individual to exercise, improve the effectiveness of exercise, enhance recovery, improve strength, speed, and endurance, reduce fatigue and increase energy. In order to gain competitive edge, athletes use dietary supplements which assert to enhance their performance [1].

In the past, sports were coupled with the masculine realm, and there was a legacy of bias against the female athletes. Since the turn of the millennium, women have been advancing gloriously in the sports world with a massive surge in the number of female athletes outstanding at the global platform [2].

Health problems in today's female athletes are due to inadequate dietary intake that leads to lack of supply of enough nutrients to support their physical demands of training. It is identified that young women athletes are also at a risk of mineral deficiency especially calcium and iron and that results in female athlete triad marked by amenorrhea, low energy availability (with or without abnormal eating pattern), and decreased bone mineral density [1,3,4]. Apart from the crucial role of macro and micronutrients and hydration an athletes' diet should also comprise of bioactive components which have been scientifically validated possessing antioxidant, immune enhancing, pain relieving, and fatigue reducing properties [5,6]. Therefore, implementation of wholesome nutrition and right supplementation to every day eating plan of the young athletes is essential to supply energy for performance, facilitate the repair and restoration of damaged tissues, enhance athletic performance and promote fitness.

Plant-based proteins are cost effective as they are less resource intensive when compared to animal protein and are emerging as a viable protein source for all [7]. Protein isolates from plant sources contain essential amino acids (EAA), branched chain amino acids (BCAA), have high digestibility factor and biological value, support postprandial muscle protein synthesis, damaged tissues repair and enhance energy production and hence are more preferred in the sports beverages and considered as a replacement of whey protein isolate [8,9,10]. Moreover, these proteins are hypoallergenic, gluten and lactose free ingredients, which meets consumers demand for natural, clean and healthy product/ingredients. They are being used in developing protein rich energy bars, spaghetti, wheat protein free baked products, and mayonnaise [11].

Owing to greater ecological sustenance and lesser production disbursements, the incorporation of plant-based protein isolates in diet designs is of utmost interest currently. The present food market has an extensive variety of plant-based proteins to choose from; on the other hand, the shortage of comparison studies between these choices stands in the way towards make the most appropriate choices. Keeping all the above points into consideration, the plant-based instant herbs enriched instant protein mix was formulated for female athletes.

2. Materials and Methods

21. Procurement of the selected ingredients

Plant-based protein isolates were ordered from the online shopping domain Amazon. Fresh & dry ingredients *viz*., vegetables (beetroot, carrots, ginger, spinach and drumstick leaves), spices (cinnamon and fenugreek), nuts and oilseeds (almonds, flaxseed, pistachio, sesame, sweet basil seeds) and brown sugar were procured from the local market of Anantapur, Andhra Pradesh.

2.2 Formulation and standardization of the instant mix

Processing of the ingredients

Vegetables and green leaves were cleaned and washed, dried at 60°C for 10-12 hours using Table top lab dehydrator (Ezidri Ultra FD1000 model) followed by coarse powdering. Oats were soaked, extraction of milk and dehydration was carried out in the laboratory. The nuts and spices were slightly roasted and powdered. All the processed ingredients were sealed in aluminium pouches and stored at room temperature.

Preparation and reconstitution of the mix

The above processed ingredients were weighed and mixed together in different permutations. The dry mix was blended well to a fine powder using a normal kitchen blender and sifted. Three variations of plant-based protein mix were developed (Table 1). Each variation comprised of protein isolates in the ratio 2:1 (variation I), 1:2 (variation II) and 1:1 (variation III). The developed variations of the mixes were reconstituted with measured amount of water, milk or oat milk, stirred and were evaluated for their acceptability.

Table 1: Composition of the developed instant protein mix (for 100 g)

Ingredients	Processing	Variation (gm)		
		I	II	III
Protein isolates (1 & 2)	---	20 +10	10+20	15 +15
Oats milk powder	Soaked, extracted, dehydrated	15	15	15
Green leaves and other vegetables	Cleaned, sliced, dehydrated, powdered	9.75	7.25	4.75
Herb mix	Cleaned, dehydrated, powdered	5	7.5	10
Edible seed gum	Soaked, extracted and dried	5	5	5
Nut and spice mix	Slightly roasted, powdered	10	10	10
Maltodextrin	---	5.25	5.25	5.25
Sweetener	---	20	20	20

Organoleptic analysis

All the variations of the developed mix were evaluated for their sensory attributes such as appearance, colour, flavour, taste and overall acceptability using the 9-point Hedonic Scale scorecard. A total number of 10 semi-trained panellists from the Department of Food and Nutritional Sciences, Sri Sathya Sai Institute of Higher Learning, Anantapur ranked their responses using the 9-point Hedonic Scale. The range of scores varied between 1 to 9 indicating "9" as extremely liked to "1" as extremely disliked. They were requested to evaluate the products with the help of the given scorecard and to present their individual acceptability about the products. The developed mixes were packed in aluminium pouch, sealed, stored at room temperature (37°C) for 4 months and used for further chemical composition analysis, *in vitro* therapeutic potential and storage stability.

2.3 Proximate constituents

The most acceptable variant (I) was further taken for analysing proximate components, selected phytochemicals *viz*., polyphenols, flavonoids, and vitamin C and *in vitro* therapeutic potential.

Moisture content was estimated in the samples using hot air oven at 100-105˚C for 6 hours. This step was repeated till constant weight was attained and the results were expressed as percent difference between fresh to dry samples. Moisture free samples were ashed using muffle furnace at 550˚C for 6 hours. The difference in the weights was recorded and expressed in percentage [12].

Total soluble sugar was estimated using phenol crystal and sulphuric acid method [13]. The samples were hydrolysed using hydrochloric acid and the supernatant was treated with 5% phenol and 96% sulphuric acid. After 20 minutes of incubation in water bath at 25-30°C the colour was measured at 490 nm. Crude fiber and total fat were estimated using FibroTron automatic fiber analysis system (FRB-4) and SoxTron automatic oil solvent extraction system (SOX-2). The difference in the weights was recorded and expressed in percentage.

Total protein in the samples was analyzed using Micro-Kjeldal apparatus (KDIGB-4M). The samples were first digested using a catalyst mixture (1:5) and concentrated sulphuric acid followed by distillation in the presence of

steam and 40% NaOH, liberated ammonia is captured by 5% boric acid solution. The bluish green distilled samples were titrated against standardized acid solution (0.1N HCl or 2N H_2SO_4).

2.4 Mineral composition
The ash samples were solubilized in acid and analyzed for calcium, iron and magnesium using standard protocols [14]. Calcium oxalate was precipitated from the ash solution and titrated against 0.01N potassium permanganate standardized solution. The calcium free filtrate was used for estimating magnesium as magnesium pyrophosphate using a gravimetric method. Iron levels were estimated using Wong's method where red color develops when ferric ions react with potassium thiocyanate and read at 540 nm.

2.5 Phytochemical profile and *in vitro* therapeutic potential
Methanolic extracts were used to determine phytochemicals (polyphenols, flavonoids and vitamin C) and *in vitro* therapeutic potential (antioxidant and anti-inflammatory) using standard protocols. Total polyphenols were estimated by Folin Ciocalteu's reagent which gives rise to blue coloration when reduced by phenolic groups at alkaline pH and measured at 540 nm [15]. Aluminum chloride assay was used to determine total flavonoids read at 510 nm [16]. DNPH method was used to analyze vitamin C in the studied samples. Red colour complex (hydrazones) formed by dehydroascorbic acid and dinitrophenyl hydrazine was measured at 540 nm [14].

Anti-oxidant and anti-inflammatory potential
Different concentrations of the methanolic extracts ranging between 2.5 to 20 µg/ml were tested for their radical scavenging and reducing ability at. The results were compared to appropriate standards *viz*., vitamin C and quercetin. Scavenging action of the extracts convert DPPH radical to a more stable product by giving an electron/hydrogen atom. Change in the colour from blue to pale yellow was measured at 517 nm [17]. Extracts reducing potential was determined by potassium ferricyanide and ferric chloride method, where Fe^{3+} converts to Fe^{2+} and forms ferric-ferrous complex. The absorbance was measured at 700 nm, higher absorbance of the reaction mixture indicates increased reducing power [18]. Total antioxidant activity was determined by measuring colored complex ferrous-tripyridyltriazine formed by reduction of Fe^{3+} to Fe^{2+} in acidic pH and measured at 593 nm [19]. Percent inhibition of the enzymes hyaluronidase and proteinase by the methanolic extract was measured at 585 nm [20] and 210 nm [21] against buffer as a blank and quercetin as positive control.

2.6 Statistical analysis
The results are presented as a mean ± standard error. The chemical data and *in vitro* therapeutic potential was subjected to One-way and two-way analysis of variance (ANOVA).

3. Results & Discussion
Ergogenic aids are materials or ingredients that enrich energy production, its usage or recovery and provide athletes with a competitive lead. Several ergogenic aids that assert to improve sports performance are used by both amateur and professional athletes [1]. With the increasing popularity of vegan diets, plant-based proteins have been shown to be a good alternative to whey protein. Moreover, they have high biological value and digestibility factor which makes them more absorbed in the blood stream faster than other sources of protein [7,8,9,10]. Considering the raised ergogenic and nutrient requirements of female athletes' simple, quick and convenient instant mix was formulated utilizing plant-based protein isolates.

Chemical composition
Table 2 presents data on chemical constituents analyzed in the developed instant mix and commercial protein mix. The developed mix showed comparable levels of crude protein (28±0 gm/100g), carbohydrate (56±0.03 gm/100 gm) and calcium (32±0.07 mg/100g) with commercial protein mix. Since we could not undertake amino acid analysis in the developed mix, the amino acid composition of the products was calculated using the standard values given by NIN, ICMR [22].

Table 3 presents the calculated amino acid composition of the developed mix. It has been proved that essential amino acids alone have the potential to stimulate muscle protein synthesis. However, amino acid composition and type of protein used for supplement formulation may differently modulate protein synthesis depending upon their digestion kinetics, presence of anti-nutritional factors. The instant mix exhibited higher content of essential amino acids than the non-essential. Role of branched chain amino acids is well known especially with respect athlete requirements, muscle mass building, and performance enhancers. The protein mix showed good amount of branched chain amino acids (mg/30 g) providing 8.13 for isoleucine, 9.83 for valine and 13.7 for leucine. The other important amino acids of the protein mix include lysine (12.01), arginine (9.61), phenylalanine (8.87), glutamic acid (5.58) and cysteine (4.16).

Table 2: Chemical composition of the instant protein mixes

Components	Plant-based protein mix	Commercial protein mix
Proximate (gm/100 gm)		
Energy (kcal)	363.90	308.15
Moisture (%)	10±0.01	19.3±2.58
Carbohydrates	56±0.03	43±0.4
Crude protein	28±0.00	31±1.09
Fat	3.1±0.00	1.35±0.25
Crude fiber	2.98±0.00	11.9±0.00
Ash	1.22±0.02	5.47±0.13
Minerals (mg/100 gm)		
Calcium	32±0.07	32±5.0
Iron	6.8±0.02	17±1.0

Magnesium	19±0.05	86.5±0
Phytochemicals (mg/100gm)		
Polyphenols	597±31.8	147±0
Flavonoids	226±0.05	22.6±0
Vitamin C	7.4±0.05	111±1

Values are mean ± SD of three replicates on dry weight basis

Table 3: Amino acid composition of the instant mix

Amino acids	100 g of Protein Mix	30g of Protein Mix (Per serving)
Histidine	11.67	3.50
Isoleucine	27.12	8.13
Leucine	45.84	13.7
Lysine	40.06	12.01
Methionine	10.23	3.06
Cysteine	13.89	4.16
Phenylalanine	29.59	8.87
Threonine	23.88	7.16
Tryptophan	10.99	3.29
Valine	32.78	9.83
Alanine	4.96	1.48
Arginine	32.05	9.61
Aspartic Acid	5.51	1.65
Glutamic Acid	18.63	5.58
Glycine	3.82	1.14
Proline	4.8	1.44
Serine	4.71	1.41
Tyrosine	11.91	3.57

Minerals play a crucial role in various metabolic and physiologic processes and optimize an athletes' performance. Processes such as muscle contraction, heart rhythm, oxygen transport etc. get accelerated during performance therefore an adequate amount of these minerals is necessary for optimal performance. During performances minerals such as calcium, iron, magnesium, potassium, selenium, zinc, and sodium get quickly depleted. Calcium and magnesium are key minerals in regulating energy metabolism, skeletal muscle contraction and relaxation. Low levels can contribute to early fatigue, nausea, and muscle cramps [23]. The calcium content of the developed and commercial mix was observed to be similar (32 mg/100g). However, bioavailability studies to be undertaken to understand the levels of available calcium from the developed instant protein mix. Iron is an element of critical importance during athletic or sports performance as body can deplete up to 5.7% of this mineral leading to fatigue, anemia and reduced endurance [23]. The levels of iron (6.8±0.02) and magnesium (19±0.05) were observed to be lower in the instant protein mix than the commercial protein mix.

Table 4 present the percent daily value for the developed mix. The nutrition facts label developed by the Food and Drug Administration, found on the foods and beverages is an important tool to make better and informed food choices which is based on one standard serving of the food. As a rule, any nutrient when providing 5% or less, out of one standard serving is low in that particular nutrient. Any nutrient providing 15% or above in a serving is considered as high in that nutrient. In the present study, products were developed keeping in mind the requirements of female athletes. The standard serving size for plant-based protein mix was fixed to be 30 gm providing 8.4 gm of protein, 9.6 mg of calcium and 2 mg of iron, providing 16.8, 0.7 and 11.1% respectively. The plant-based protein mix was standardized for its reconstitution with milk, water, oat milk and water: milk with various ratios of 1:1, 2:1 and 1:2.

Table 4: Percent daily values of plant-based protein mix

Nutrition Facts	
Serving size (30gm)	
Amount per serving	
Calories 109.17	Calories from Carbohydrates 67.2 Calories from Fats 23.9 Calories from Proteins 33.6
% Daily value*	
Total carbohydrates 16.8g	6.1%
Fiber 0.89g	3.1%
Protein 8.4g	16.8%
Total fat 0.93g	3.3%
Calcium 9.6mg	0.7%
Iron 2.0mg	11.1%
Magnesium 5.7mg	1.35%
Zinc 1.2mg	10.9%
Vitamin C 2.22mg	2.4%
*Percent daily values are based on a 2000 calorie diet	

The instant protein mix was studied quantitatively for phytochemicals such as polyphenols, flavonoids, and vitamin C (Table 2). Polyphenols have a wide range of biological activities which acts as antioxidant, anti-carcinogenic and anti-mutagenic properties and possess the ability to modify gene expression. The formulated protein mix exhibited higher levels of polyphenols and flavonoids content (597±31.8 and 226±0.05 mg/100 g) when compared to commercial protein mix (147±0 and 22.6±0). The developed products could exhibit high levels of these bioactive components due to the corporation of functional ingredients such as dehydrated vegetables and edible flower. Role of vitamin C as an antioxidant and immune enhancer has been well established. Highest levels of the vitamin were observed in commercial mix which could be due to fortification of the mix with ascorbic acid.

The data on *in vitro* therapeutic potential of the developed

and commercial protein mixes is presented in Table 5. The developed instant protein mix extracts exhibited significant ($p<0.05$) anti-oxidant and anti-inflammatory action. Quercetin exhibited lowest IC_{50} value followed by plant-based protein mix and commercial protein mix. A similar trend was observed with ascorbic acid standard exhibiting higher ferric ion reducing potential than the protein mixes. The significant therapeutic potential has been attributed to the presence of different polyphenols, flavonoids, vitamin C, carotenoids and other present phytochemicals in the instant mix.

Table 5: *In vitro* therapeutic potential of the developed and commercial protein mix

Sample	IC_{50} value			FRAP (µM)	Reducing power (nm)
	DPPH RSA	Anti-hyaluronidase action	Anti-proteinase action		
Plant-based protein mix	7.77 ± 0.01	59.52 ± 0.06	5.34 ± 0.03	2.35±0.04	0.59±0.02
Commercial protein mix	8.91 ± 0.03	63.45 ± 0.11	5.53 ± 0	3.07±0.03	0.52±0.02
Std. Quercetin	7.70 ± 0.05	29.41 ± 0	5.01 ± 0.01	-	-
Std. Ascorbic acid	-	-	-	3.39±0.02	0.61±0.04

Values are mean ± SD of three replicates on dry weight basis

4. Conclusion

The natural ingredients used in the instant mix are rich source of essential nutrients and bioactive components, making it a suitable pre and post workout product for the athletes as they meet the requirements, reduce oxidation and inflammation. The mix is a great supplement for muscle building and recovery. The developed protein mix can also be utilized by healthy population. Water activity of the instant mix was 0.54 at the end of 4 months' storage period at RT indicating product stability. The product is cost to prepare 100 gm of mix is Rs. 15 which is significantly lower when compared to a commercial plant-based protein mix.

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