

Production of a Probiotic-based Immuno-booster Nutraceutical Drink for Immunocompromised Individuals and *in vitro* Analysis of the Drink

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ABSTRACT

Background and Aim: Immunocompromised individuals suffer from a disrupted immune system that leads to inflammation, infection, delayed recovery and other physiological diseases. Thus, this study aimed to develop an immune-booster nutraceutical drink from plant-based materials, supplement it with probiotics and analyze its nutritional efficacy and potential health benefits.

Materials and Methods: Immune-booster nutraceutical drink was produced by combining the plant components tender coconut water, ginger, lemon and *Aloe vera* as well as other materials. Further, the probiotic *Lactobacillus acidophilus* was added to the drink and its nutritional value, including glucose, protein, carbohydrate, lipid and iron, was calculated. The shelf life and pH were analyzed to verify its quality. This was analyzed using gas chromatography mass spectroscopy to identify the phytochemicals. Molecular docking of the compounds reported using gas chromatography mass spectroscopy was carried out against IgE to identify the best compound with immunity. Cytotoxicity of immune-booster nutraceutical drink to human leukemia monocytic cell line (THP-1) cells was identified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and the interleukin 6 levels were quantified using enzyme-linked immunosorbent assay.

Results and Conclusion: Immune-booster nutraceutical drink, an immune-booster nutraceutical drink supplemented with *Lactobacillus acidophilus* (probiotic), was prepared and the nutritional value of it was detected to possess decreased glucose (0.07 mg ml⁻¹) and increased carbohydrate (8.96 mg ml⁻¹), protein (0.62 mg ml⁻¹), lipid (5.60 mg ml⁻¹) and iron (2.22 mg ml⁻¹). The acidic pH of 3.9 and the decreased microbial load (5.9 × 10⁹ CFU ml⁻¹) simultaneously verified the active probiotic and increased shelf life. Gas chromatography mass spectroscopy detected 58 phytochemicals in immune-booster nutraceutical drink and their molecular docking verified the immunity-increasing characteristics of nicotinamide against IgE, with a binding energy of -3.9 kcal mole⁻¹. Cytotoxicity showed non-toxic nature with IC₅₀ (508.1 µg ml⁻¹) and decreased interleukin 6 with IC₅₀ (544.5 µg ml⁻¹). The developed immune-booster nutraceutical drink, supplemented with probiotics, possessed increased nutritional values, without toxicity or side effect and could be studied further to boost immunity in immunocompromised individuals.

Highlights:

- Immunocompromised individuals suffer from a disrupted immune system that leads to inflammation, infection, delayed recovery and other physiological diseases
- An immune-booster nutraceutical drink from plant-based materials, containing probiotics may help immune system

What this article adds:

- An immunity boosting nutraceutical drink was produced by combining positive health attributes exerting plant components and probiotic *Lactobacillus acidophilus*.
- Immunity boosting nutraceutical drink demonstrated improved nutritional value, pH and shelf life.
- Immunity boosting nutraceutical drink was reported as non-toxic to normal cell lines.
- Developed drink decreased the levels of inflammatory cytokine of IL-6.

1. INTRODUCTION

The current urbanized world increases the exposure to multiple microbes and toxic compounds present in the environment. Hence, the immune system fights against the foreign invaders and protects the human body from bacterial, fungal and other infections [1-3]. The immune system plays a vital role in preserving the health of individuals [4,5]. This needs supplementation of the human body with recommended nutritional components such as vitamins and minerals, which boost immunity [6,7]. Several plants such as ginger, garlic, alma, Tulsa, drumstick and neem contain phytochemicals that enhance immunity, without side effects [8,9]. In addition to increasing immunity, these possess increased antimicrobial and antioxidant characteristics [10,11]. Compared to healthy foods, beverages and drinks are further preferred due to their accessibility, storage and transportation facilities. Additionally, these are enriched with high nutritional values and favor sensory qualities such as flavor, taste and feel appealing [12,13].

Nutraceutical drinks are preferably used by common people as alternatives to medicine to prevent diseases and minimize side effects [14-16]. An immune-booster drink prepared with a combination of honey, lime juice and red ginger showed increased vitamin C and antioxidant levels [17,18]. Citrus fruits, ginger, garlic, spinach, bell pepper, pomegranate and kiwi are well known to increase immunity [19,20]. Lemon possesses increased polyphenols, tannins, terpenes and other phytochemicals [21,22]. Tender coconut water is addressed as a nutritional drink, as it is rich in vitamins, minerals, sugars and amino acids and as a low-calorie electrolyte-rich hydrating drink [23]. The use of tender coconut water in producing tender coconut lassi, coconut water kefir and souffle has been implemented to increase nutritional values [25,26]. *Aloe vera* consists of increased water and minerals, especially magnesium, potassium and calcium that facilitate hydration and preserve electrolyte balance [27,28]. *Aloe vera* is known for its variable health benefits and its incorporation with the pulp of pineapple in beverage preparation showed improved sensory and physiochemical qualities [29,30]. Although all these plant components are assessed for their values as a nutritional drink, their appropriate combinations in drinks are not widely reported.

Probiotics are bacteria derived from foods that secrete active compounds such as short-chain fatty acids, vitamins

and antioxidants for their metabolism. These compounds show a direct effect on the consumer's health [31,33]. Within multiple probiotics, including *Enterococcus*, *Bifidobacterium*, *Lactobacillus* and *Lactococcus*, *Lactobacillus* inhabits the gastrointestinal tract (GIT) predominantly, decreases adhesion of pathogens, increases immunity and preserves functioning of the intestinal barrier [34,35]. Especially, *L. acidophilus* decreased the fat content in ice creams [36,37]. Although multiple studies have been reported to incorporate probiotics in nutraceutical drinks, the combination of coconut water, lemon, ginger and *Aloe vera* is still unique and the supplementation of *L. acidophilus* is reported in the present study. Hence, this study aimed to produce an immune-booster nutraceutical drink (IND) by combining tender coconut water, ginger, lemon juice and *Aloe vera* at appropriate concentrations and the supplementation of probiotics (*L. acidophilus*) to produce a nutraceutical drink [38,39]. Its nutritional characteristics such as total energy value, glucose, carbohydrate, protein, lipid and iron were estimated and physical characteristics such as shelf life and pH were calculated. Compounds present in IND were analyzed using gas chromatography mass spectroscopy (GCMS) and their efficacy to increase immunity was assessed using docking technique [40,41]. Furthermore, the drink cytotoxicity to normal blood cells was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Interleukin 6 (IL-6) was quantified to identify the decrease of inflammatory cytokines [42,43]. Thus, the present study facilitated producing an efficient immunity boosting nutraceutical drink that improved nutritional values, shelf life and pH, decreased toxicity and potentially decreased IL-6 as an inflammatory cytokine.

2. MATERIALS AND METHODS

2.1. Materials and Cell lines

Glucose, 1 M ammonium thiocyanate, 1 M HCl, sulfuric acid and iron sulfate were purchased from HiMedia, India. Anthrone reagent, Bradford's reagent, bovine serum albumin (BSA), sulfophosphoric-vanillin acid and cholesterol were purchased from Sigma Aldrich, USA. Chloroform and methanol were purchased from SRL, India. The 96-well plate was purchased from Tarsons, India. Beakers and tubes were purchased from Borosil, India. The enzyme-linked immunosorbent assay (ELISA)



plate reader, vortex mixer, mantle heater and crucible were used as well.

The human leukemia monocytic cell line (THP-1) cell lines were purchased from the National Centre for Cell Science (NCCS), Pune, India. Roswell Park Memorial Institute (RPMI) media, antibiotic solution and fetal bovine serum (FBS) were purchased from Gibco, USA. The MTT (5 mg ml⁻¹) and dimethyl sulfoxide (DMSO) were purchased from Sigma, USA. Moreover, 1× PBS was purchased from SRL, India. The 96-well tissue culture plate and wash beakers were purchased from Tarsons, India.

2.2. Methodology

2.2.1. Immune-Booster Nutraceutical Drink Preparation

Plant components were ground using mortar and pestle to collect the extract and the raw materials were purchased from a local store, Tiruchirappalli, India [21, 49, 50]. The extracts of the plant components, including tender coconut water (60 ml), ginger (2 ml), lemon juice (3 ml) and *Aloe vera* (6 ml), and other raw materials, including L-glutamine (3 g), marine collagen (4 g), zinc citrate (5-10 mg), B-complex vitamins (1/10 of the capsule) and sodium benzoate (0.05 mg), were mixed and made up to 100 ml to produce the immune-booster nutraceutical drink.

2.2.2 Supplementation of an Immune-Booster Drink With Probiotic Culture

The *L. acidophilus*, a probiotic culture, was purchased from MTCC, India [22,51]. The organism was subcultured and preserved in a nutrient media until reaching 0.5 OD of McFarland concentration. The culture was then centrifuged and 0.1 g of the culture pellet was added to 100 ml of the immune-booster nutraceutical drink.

2.2.3 Glucose Assessment

Standard glucose solution (5 mg ml⁻¹) was serially diluted using eight tubes and 500 µl of stock solution and 500 µl of distilled water (DW). The IND (100 µl) with 400 µl of DW was transferred into a fresh tube. To all the tubes, 200 µl of Benedict's reagent were added. The tubes were heated for 10 min and the contents were transferred to a 96-well plate triplicate. The absorbance was measured at 595 nm. A standard graph was plotted using GraphPad Prism for the values of the standard solution; based on which, the

accurate quantity of glucose in the sample was estimated [23].

2.2.4 Lipid Assessment

Standard cholesterol solution (10 mg ml⁻¹) was prepared by serial diluting of chloroform:methanol (2:1 ratio) using eight tubes. The IND (100 µl) was dissolved in 400 µl of water and assessed for its lipid concentration in a fresh tube. To the tubes, 100 µl of concentrated sulfuric acid were added and incubated at 90 °C for 10 min. After incubation, the tubes were cooled and 50 µl of sulfo-phosphoric-vanillin acid was added. The absorbance of the tubes was measured at 540 nm after the development of purple color. A standard graph was plotted using GraphPad Prism for the values of standard solution; based on which, the accurate quantity of lipid in the sample was estimated [25].

2.2.5 Carbohydrate Assessment

Glucose stock solution (5 mg ml⁻¹) was prepared and serially diluted using 500 µl of each stock solution and DW. The IND (100 µl mixed with 400 µl of DW) was transferred into a fresh tube. To the contents, 200 µl of 75% sulfuric acid were added. Then 400 µl of anthrone reagent were added to the mixture, vortexed well and boiled at 100 °C for 15 min. The absorbance was measured at 578 nm using microtiter plate. A standard graph was plotted using GraphPad Prism for the values of the standard solution; based on which, the accurate quantity of carbohydrate in the sample was estimated [34].

2.2.6 Protein Assessment

The BSA standard (5 mg ml⁻¹) was prepared and serially diluted (500 µl) with DW (500 µl). The IND (500 µl) was added to a fresh tube and 1500 µl of Bradford's reagent were added to all the tubes and incubated for 10 min. After the observation of the blue color change, the spectrometric reading was carried out at 595 nm. A standard graph was plotted using GraphPad Prism for the values of standard solution; based on which, the accurate quantity of protein in the sample was estimated [14].

2.2.7 Iron Assessment

Iron assessment was carried out using calorimetric ammonium thiocyanate assay. For the assessment, the IND sample was transferred into a crucible and heated to 400 °C. The resulting ash was mixed with 1 M HCl (10 ml) and



DW (5 ml). The mixture was filtered and used for the assessment. Moreover, 10 ml of the iron sulfate standard solution (50 mg ml⁻¹) were serially diluted using eight tubes. The IND sample (10 ml) was transferred into a fresh tube. To all the tubes, 1 M ammonium thiocyanate (10 ml) was added and incubated [15 min, room temperature (RT)]. After development of red color, absorbance was measured at 510 nm using UV-vis spectrophotometer. GraphPad Prism was used to plot the standard curve and the total iron was expressed as mg of iron equivalents per 100 g of dry mass [18].

2.2.8 Assessment of Total Energy Value

Total energy of the IND was calculated using Eq. 1:

$$\text{Energy (kcal)} = 4 \times (\text{proteins} + \text{carbohydrates in grams}) + 9 (\text{mass of fat in grams}). \quad \text{Eq.1}$$

2.2.9 Shelf Life

Shelf life of the IND was calculated by enumerating the microbes in the sample. The IND was serially diluted and 100 µl of 10² dilutions were plated onto LBS (*Lactobacillus* selective agar) media. The media were prepared by dissolving 1 g of peptone, 0.5 g of yeast extract, 2.0 g of dextrose, 1.5 g of sodium acetate, 0.6 g of potassium dihydrogen phosphate, 0.2 g of ammonium citrate, 0.1 g of polysorbate 80, 0.05 g of magnesium sulfate, 0.012 g of manganese sulfate, 0.003 g of ferrous sulfate and 1.5 g of agar in 100 ml of DW. The pH decreased to 5.5 by adding glacial acetic acid (AA). The dissolved contents were autoclaved (118 °C, 15 min, 12 lbs), mixed well and poured onto 100 mm Petri plates, while the media was in a molten state. The diluted drink (10²) was plated over the media using spread plate method. The plates were then incubated at 37 °C for 24 h and the colonies were counted [27]. The colony-forming units (CFU) were calculated using Eq. 2;

$$\text{Energy (kcal)} = 4 \times (\text{proteins} + \text{carbohydrates in grams}) + 9 (\text{mass of fat in grams}). \quad \text{Eq. 2}$$

2.2.10 Assessment of pH

The pH of the IND was analyzed using portable electronic pH meter (manufacturer, country). A known pH buffer was used to calibrate the pH meter and change in pH of the sample at constant temperature was reported [23,52].

2.2.11 Gas Chromatography Mass Spectroscopy Analysis

The GCMS included GCMS-QP2010SE (Shimadzu gas chromatograph coupled to a mass detector). The IND sample (1 ml) was injected and the parameters of the instrument were set as follows. Capillary column [24,53], 250 µm; oven temperature, 250 °C; injection port temperature, 280 °C; helium flow rate, 1 ml/min; ionization voltage, 70 Ev; sample injection in split mode, 1:10 ratio; mass spectral range, 40–450 MHz; and source temperature, 350 °C. The detected compounds were used for molecular docking.

2.2.12. Molecular Docking

Nicotinamide (a phytochemical) structure was downloaded from the PubChem website (PubChem CID: 936) in 3D SDF format. The downloaded structure was visualized using BIOVIA Discovery Studio software and converted to PDB format. The ligand (nicotinamide) was prepared using Autodock v.4.2.6 software. The compound merged four non-polar hydrogens and detected two rotatable bonds. The PDB format was converted to PDBQT using AutoDock v.4.2.6 software. Moreover, IgE (PDB ID 1F6A) was selected based on the X-ray diffraction (XRD) method. The PDB format of the protein was retrieved from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) and visualized and optimized using BIOVIA Discovery Studio software. The target protein was then optimized by deleting the heteroatoms and water molecules. The polar hydrogen was added and the active sites were reported. The attributes of xyz were identified as 1F6A 49.140539, 104.809286 and 175.210177, respectively. The protein format was converted from PDB to PDBQT [Protein Data Bank, Partial Charge (Q) and Atom Type (T)] using Auto Dock v.4.2.6 software. The optimized ligand and protein were used in docking after preparing conf. file. Figure 1 shows 3D structure of the ligand, protein and optimize assessment. Auto Dock Vina software was used by adding information to the command prompt. Topmost configuration of the complex was interpreted using BIOVIA Discovery Studio [25,54].



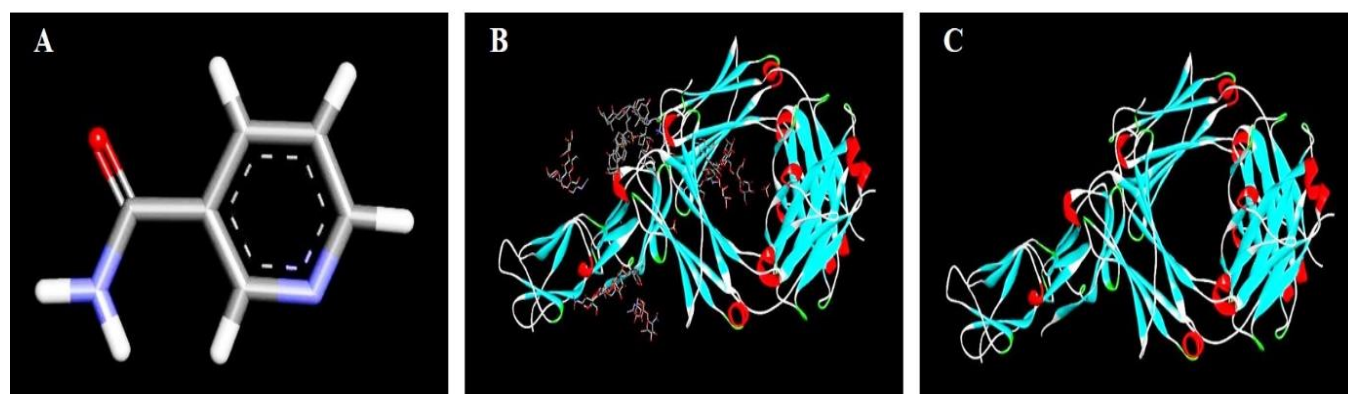


Figure 1. The 3D structures of the ligand, protein and optimized protein

Note: A represents 3D structure of the ligand, B shows 3D structure of the protein, and C represents their optimized structure.

2.2.13 Cytotoxicity Analysis Using MTT Assay

Cytotoxicity of the IND to THP-1 cell lines was assessed using MTT assay [26,55]. The cells were cultured in liquid RPMI media, supplemented with 10% serum FBS and 100 µg/ml of penicillin/streptomycin and set under 5% CO₂ atmosphere at 37 °C. The cultured cells were collected in a 15-ml tube by trypsinization [27,56]. The cells of 200 µl were plated in 96 well plates, at a density of 1×10^5 cells/ml/well. The plates consisted of RPMI media were supplemented with 10% FBS and antibiotic solution. The plates were then incubated at 37 °C for 24–48 h. Wells were washed with sterile PBS, treated with various concen-

trations of IND sample and reincubated (37 °C, 5% CO₂, 24 h). Moreover, 10 µl of MTT (5 mg ml⁻¹) were added to the tubes and incubated for 2–4 h to visualize purple precipitates using inverted microscope. After incubation, the media and MTT were aspirated off and washed with 1× PBS. The DMSO (100 µl) was added to the plates and the plates were agitated for 5 min to dissolve formazan crystals. The absorbance of the wells was measured using microplate reader (Thermo Fisher Scientific, USA) at 570 nm. The cell viability proportion and IC₅₀ were calculated using GraphPad Prism and Eq. 2:

Formula cell viability % = test OD / control OD × 100Eq. 2

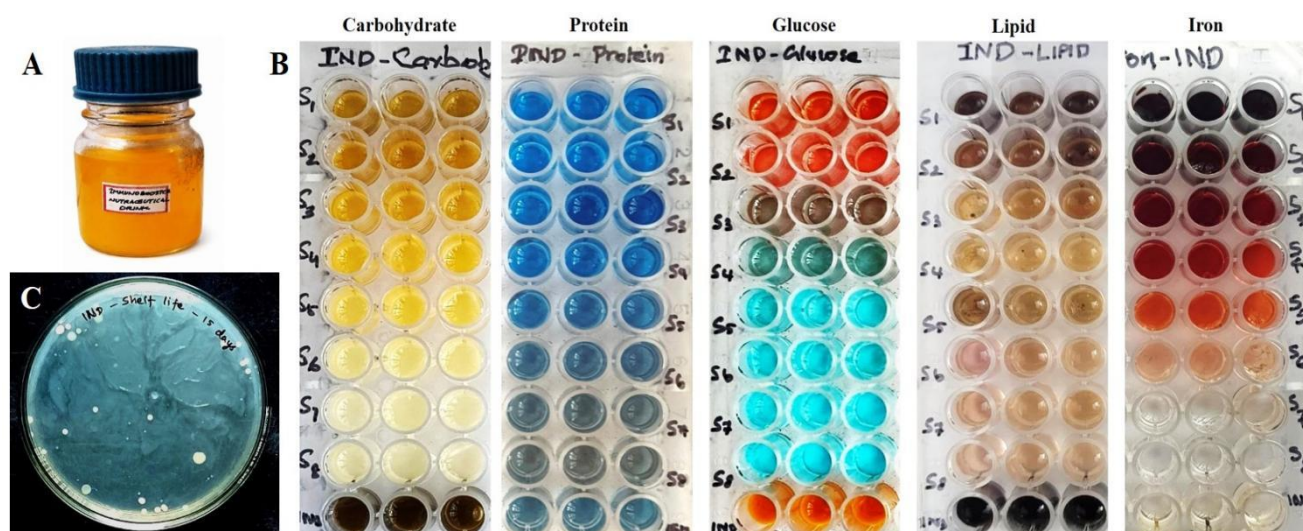


Figure 2. Photographic representation of the produced nutraceutical drink and its nutritional value and probiotic level.

Note: 2A represents the produced nutraceutical drink, 2B shows the assessment of nutritional value of Immune-booster nutraceutical drink and 2C represents the Petri plate showing live microbial cells in Immune-booster nutraceutical drink.



2.2.14 Enzyme-Linked Immunosorbent Assay-interleukin 6

Similar to the MTT assay, THP-1 cells were cultured and harvested to identify the effect of IND on IL-6 release [28,57]. The cells were plated in RPMI media using 96-well plate, PBS washed, treated with the IND sample and incubated, following a similar procedure in the MTT assay. After incubation, the cells were washed with PBS, stimulated with 50 ng/ml of LPS and incubated (37 °C, 24 h, 5% CO₂). Cell-free supernatant was collected by centrifugation (1500 rpm, 5 min) and IL-6 was estimated using ELISA method based on a protocol by Invitrogen, USA. The absorbance was measured at 450 nm using microplate reader (Thermofisher Scientific, USA).

2.2.15 Statistical Analysis

The statistical analysis and the calculation of IC₅₀ values were carried out using GraphPad Prism software v.8.0, USA.

3. RESULTS AND DISCUSSION

The IND was prepared of plant extracts and other raw materials. The plant components, including tender coconut water, ginger, lemon juice and *Aloe vera*, have been known for their health benefits for centuries [29, 44, 58]. Thus, an IND from these combinations has been produced. The incorporation of probiotics in nutritional drinks is known to provide bioactive compounds. Thus, combining nutritional components with probiotics increases the health benefits by providing health attributes from the two sources [30,59], Figure 2A shows the prepared nutraceutical drink, supplemented with probiotics. Other studies have reported the use of these plant components in the preparation of drinks; however, a combination of all these components and the incorporation of probiotics, has not been reported. Increased concentration of limejuice with 4% showed the highest antioxidant activity (IC₅₀ of 11.21 ppm) and vitamin C content (9103.88 µg ml⁻¹), compared to the decreased limejuice concentration [15, 31, 45]. Another beverage with coconut water (80%) and orange juice (20%) showed increased antioxidant activity due to higher levels of flavonoids, ascorbic acid, hesperidin, Gallic acid, cinnamic acid and phenolics [19, 32, 46]. Ginger possesses increased immunomodulatory, antioxidant and anti-inflammatory characteristics. Similarly, the combination of

red ginger with lime and honey was reported to possess increased antioxidant activity and immune efficiency due to the presence of vitamin C [15, 33, 47]. *Aloe vera* has recently increased metabolic health and immunity by increasing hydration, antioxidant activity and gastrointestinal comfort [8, 34]. Hence, it supports the current study in combining coconut water, ginger, *Aloe vera* and lemon juice, a vitamin C containing compound. Simultaneously, the active nature of the probiotics was verified by viable colony count that showed 5.9×10^9 CFU/ml after 24 h of incubation. As already discussed, the probiotic presence increases the bioactive compounds, increases drug transfer and improves gut health. Probiotics that include lactic acid bacteria (LAB) preserve gut microbiota and improve immunity in people with weakened immune systems [27, 35]. A probiotic (*L. acidophilus*) drink produced by blending pineapple juice and whey demonstrated the highest sensory score and increased digestion [17, 36]. Similarly, addition of probiotics such as *B. longum*, *L. acidophilus* and *L. casei* into health drinks of carrot juice, beetroot juice, ginger and lemon showed increased protein contents and decreased carbohydrates. This increased protein, phenol, flavonoid and antioxidant activities [6, 37]. Thereby, all these studies verify that supplementation of probiotics with nutritional compounds improves the health attributes, particularly, gastrointestinal flora regulation and immunity development [38,48] in immunocompromised individuals.

3.1 Assessment of Nutritional Values of The Prepared immune-Booster Nutraceutical Drink

Nutritional values of the nutraceutical drinks seem to improve health conditions and treat diseases [39,1]. Increased sugars increase glucose spike and it is important to decrease the glucose content of drinks to prevent diabetes such as issues [20,40, 49]. Similarly, the sugar content of the current study included the lowest value in the study. Levels of protein, carbohydrates and iron need increases to improve the health of immunocompromised individuals. Likely, the produced IND contained increased nutritional components such as 8.969 mg/ml carbohydrate, 0.621 mg ml⁻¹protein, 0.07486 mg ml⁻¹glucose, 5.608 mg ml⁻¹lipid, 2.226 mg ml⁻¹iron and 88.832 kcal mole⁻¹ total energy value (Table 1). Note: Mean ±SD was calculated for the tests carried out in triplicates. The SD refers to standard deviation, mg/ml refers to milligram per milliliter, kcal/mole indicates kilo calories per mole, CFU / ml shows colony forming units per milliliter.



Table 1. Nutritional values and physical parameters of the produced immune-booster nutraceutical drink

S. No	Parameter	Value (Mean $\pm$ SD)	Units
	Carbohydrate	8.969 $\pm$ 0.10	mg/ml
	Protein	0.621 $\pm$ 0.01	mg/ml
	Sugar / glucose	0.07486 $\pm$ 0.03	mg/ml
	Lipid	5.608 $\pm$ 0.07	mg/ml
	Iron	2.226	mg/ml
	Total energy value	88.832	Kcal/mole
	pH	3.9	--
	Shelf life	5.9 $\times 10^9$	CFU/ml

Figure 2B represents the 96-well plate showing assessment results of the nutritional values. A nutraceutical beverage containing Roselle, *Aloe vera* and guava showed increased iron content, similar to the study. The increased iron in the study could be attributed to the increased iron content of *Aloe vera* [22, 41]. Lemon and mustard-based beverages showed improved physicochemical characteristics, antidiabetic, antioxidant and hypocholesterolemia activities [5, 42]. The coconut water contained water (95.5%), carbohydrate (4%), fat (0.1%), iron (0.5%), amino acids, vitamins and minerals [43,50]. Energy booster drinks from beetroot showed 297.5 g 1000 ml⁻¹ carbohydrate, 10.95 g1000 ml⁻¹ lipid and 40.25 g1000 ml⁻¹protein [44,3], which were comparatively less than those in the current study. All this nutrition could be a reason for the increased nutritional value of the IND drink. As LAB are capable of mediating fermentation, they improve human health [45,11]. Combining these nutritional values with the probiotics increases gut health and immunity of immunocompromised individuals.

The pH is responsible for the maintenance of acidic or basic conditions of the body. The pH of the drink was detected as 3.9 and the acidic pH showed decreased microbial growth (5.9 $\times 10^9$ CFU ml⁻¹) and extended shelf life. Figure 2C shows the decreased microbial load, which indicated increased shelf life and active probiotics. The values of the parameters are depicted in Table 1. The acidic pH of the drink could be attributed to the presence of vitamin C in

lemon juice [46,3]. Multiple studies have reported acidic pH of the drink [48,9]. The acidic pH, active probiotic and increased shelf life show the better quality of the product.

3.2 Gas chromatography Mass Spectroscopy Analysis of Immune-Booster Nutraceutical Drink

The GCMS analysis was carried out to identify changes in the volatile composition of the products during preparation and the phytoconstituents [43,49]. The GCMS analysis of IND showed the presence of 58 predominant compounds; within which, but-3-enyl-(E)-methylbut-2-enoate, benzoic acid, cyclopropane carboxylic acid, 1-amino, DL-proline,5-oxo, nicotinamide, caffeine, 9H-purin-6-amine, and N,9,dimethyl was detected predominantly. All these compounds were assessed for their efficacy to possess immunity development. Peaks showing the presence of predominant compounds are depicted in Figure 3 and the compounds are listed in Table 2. Similar to the current study, a nut-based beverage detected nearly 70 compounds, including alcohols, ketones and aldehydes [23, 50]. A fermented nutraceutical beverage from *Haematocarpus validus* showed the presence of active antioxidant compounds such as anthocyanins, polyphenols and flavonoids [28,51,56]. However, no similar compounds detected in the current study were identified. This highlighted the produced nutraceutical drink, possessing increased immunity-developing characteristics



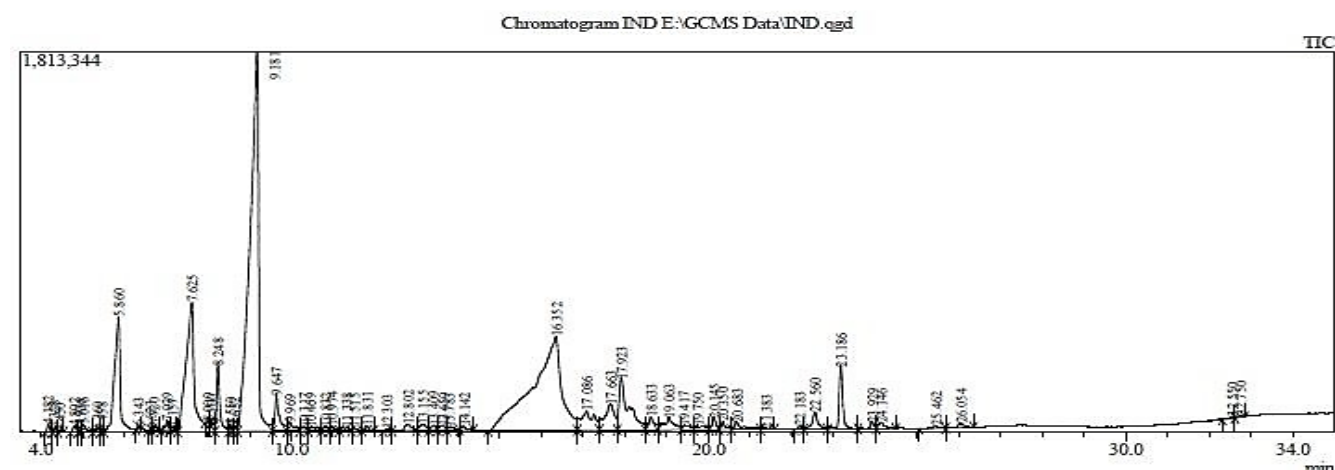


Figure 3. Gas chromatography mass spectroscopy chromatogram of the immune-booster nutraceutical drink

Table 2. Compounds identified from the immune-booster nutraceutical drink using gas chromatography mass spectroscopy

S. No	Retention time	Area	Area %	Compound name
	4.182	185377	0.22	Pyrazine, 2,5-dimethyl-
	4.328	98888	0.12	Tetrahydropyran
	4.45	29895	0.04	3-Amino-s-triazole
	4.802	205918	0.24	2,5-Furandione, dihydro-3-methylene-
	4.936	106466	0.12	2-Furanmethanol, 5-methyl-
	5.016	247724	0.29	2-Furancarboxaldehyde, 5-methyl-
	5.36	96360	0.11	2-Dimethylsilyloxypentane
	5.458	36699	0.04	Acetic acid, TBDMS derivative
	5.86	5727949	6.72	But-3-enyl (E)-2-methylbut-2-enoate
	6.343	70947	0.08	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (. +/-)-
	6.623	22908	0.03	Butanal, 2-methyl-
	6.72	127667	0.15	2,5-Dimethylfuran-3,4(2H,5H)-dione
	7.029	296462	0.35	4-Amino-6-hydroxypyrimidine
	7.157	73358	0.09	Acetamide, 2-(2-thiophenyl)-N-ethyl-N-butyl-
	7.625	8989727	10.55	Cyclopropanecarboxylic acid, 1-amino-
	8	36902	0.04	(Z)-2-Methoxyimino-3-oxobutyric acid, 1,1-dimethylethyl ester
	8.1	35415	0.04	2-Butenedioic acid (Z)-, monobutyl ester
	8.248	1750824	2.05	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
	8.55	129910	0.15	2-(tert-Butyldimethylsilyl) thiazole
	8.652	152098	0.18	Pentanoic acid, 4-methyl-
	9.181	25420544	29.83	Benzoic acid
	9.647	1321325	1.55	3-Hexene
	9.969	416175	0.49	2(1H)-Pyridinone, 5-methyl-
	10.317	173545	0.2	Picolinamide
	10.469	256025	0.3	1H-Imidazo[1,2-b] pyrazole, 2,3-dihydro-
	10.832	189360	0.22	3-Penten-1-ol, (E)-
	10.974	202046	0.24	Oxazole, 2,4-dimethyl-
	11.338	212770	0.25	Pyrazine, 2-ethyl-6-methyl-
	11.515	100622	0.12	1-Methylpyrrolo[1,2-a] pyrazine
	11.831	106093	0.12	4-Hydroxy-7-methyl-1,8-naphthylidene
	12.303	65750	0.08	Propane, 2-isocyanato-2-methyl-
	12.802	373654	0.44	Niacinamide
	13.155	349382	0.41	2-(Hexyloxy)acetic acid
	13.409	224867	0.26	Diethylamine, 2,2'-dichloro-
	13.65	236856	0.28	1,5-Heptadien-4-ol, 3,3,6-trimethyl-
	13.783	175706	0.21	Cyclobutene-3,4-dione, 1-dimethylamino-2-hydroxy-
	14.142	141751	0.17	6-Heptenoic acid



16.352	21462170	25.18	DL-Proline, 5-oxo-
17.086	1960878	2.3	Norbelladine
17.663	2038061	2.39	Propane, 2-isocyanato-2-methyl-
17.923	3850734	4.52	9,9-Dimethyl-2-oxabicyclo [4.2.1] nonan-3-one
18.633	674119	0.79	Pyrrole-2-carboxaldehyde, 1-[1-(1-adamantyl) ethyl]-
19.063	1013394	1.19	Caffeine
19.417	301114	0.35	Heptanal
19.75	309480	0.36	Pyrrolidine, 1-methyl-
20.145	420356	0.49	cis, trans-4-n-Propyl-3-oxabicyclo [4.4.0] decane
20.35	293112	0.34	n-Hexadecanoic acid
20.683	522878	0.61	2-(Dimethylamino)-3-methyl-1-butene
21.383	102819	0.12	Benzoic acid 2-methylpentyl ester
22.183	83830	0.1	2H-Inden-2-one, octahydro-
22.56	791098	0.93	3H-Cyclopenta[c]pyridazin-3-one, 2,5,6,7-tetrahydro-
23.186	1860480	2.18	9H-Purin-6-amine, N,9-dimethyl-
23.929	250873	0.29	3-Pyrrolidin-2-yl-propionic acid
24.146	350473	0.41	3-Cyclopentene-1-ethanol, 2,2,4-trimethyl-
25.462	144605	0.17	cis, trans-4-methyl-3-oxabicyclo [4.4.0] decane
26.054	265376	0.31	Cyclohexanol, 3-ethenyl-3-methyl-2-(1-methylethenyl)-6-(1-methylethyl)-, [1R-(1.alpha.,2.alpha.,3.beta.,6.alpha.)]-
32.55	78786	0.09	Diosgenin benzoate
32.75	61470	0.07	18-Pentatriacontanone

3.3 Molecular docking

Molecular docking facilitates the prediction of bonding between ligand and protein. It helps identify binding sites and optimize peptide activity [45,52,57]. Although IgE plays significant role in allergic response, it contributes to pathogen defense, immune regulation and antigen presentation [34,53,58]. Thus, identifying a compound that binds to IgE facilitates identification of phytochemical that possess immunomodulatory roles. Within the compounds docked against IgE, nicotinamide showed binding energy of $-3.9 \text{ kcal mole}^{-1}$. The docked complex formed four hydrogen

bonds and one hydrophobic Pi-Pi alkyl bond with the amino acids. Figure 4 shows 2D and 3D structures of the docked complex and Figure 5 shows the interactions of the bonds with protein and ligand. The proteins of β -conglycinin, glycinin, catechin and epigallocatechin-3-gallate showed decreased binding efficacy against IgE [16,54,59]. However, the docking energy between nicotinamide and IgE was not reported. Further, no studies have reported the molecular docking of the nutraceutical drink against IgE and the current study was still the first, as far as the authors' knowledge is concerned [60,61].

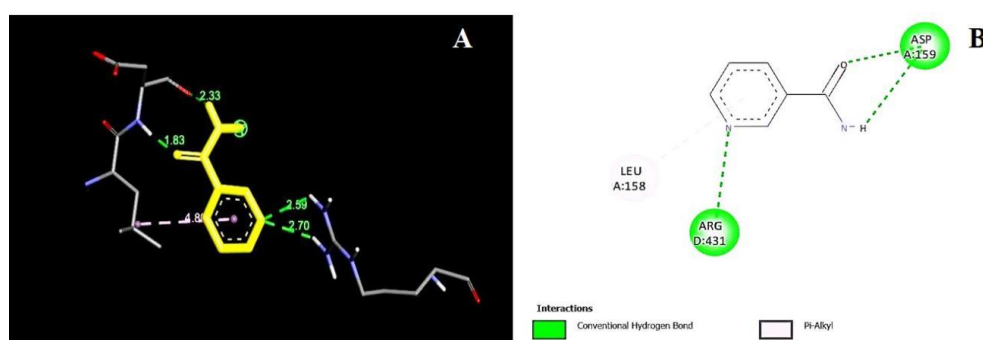


Figure 4. The 3D and 2D structures of the docked complex of nicotinamide (ligand) and IgE (immunity protein)
Note: A shows 3D structure of the docked complex, and B represents 2D structure of the docked complex



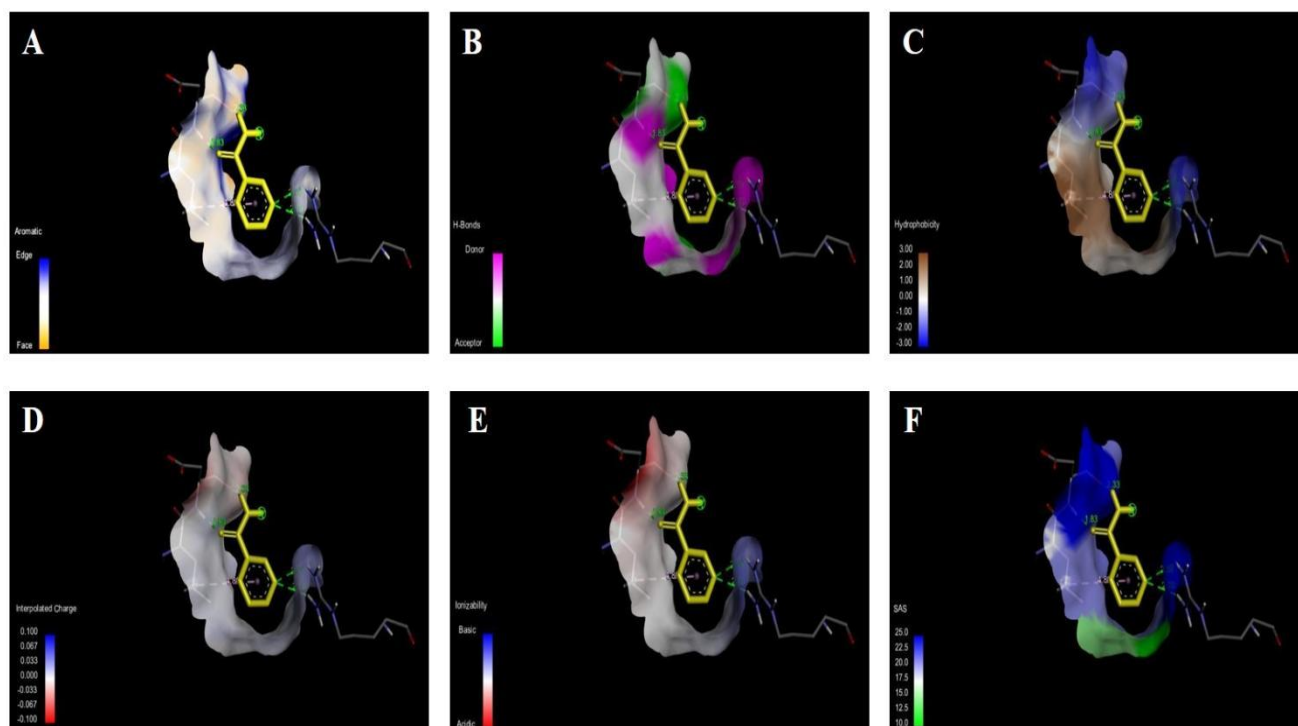


Figure 5. Bond interactions between the protein and ligands

Note: A shows the aromatic bonds, B represents H bond interactions, C shows hydrophobicity, D demonstrates charge, E shows the ionizability and F represents the solvent accessible surface

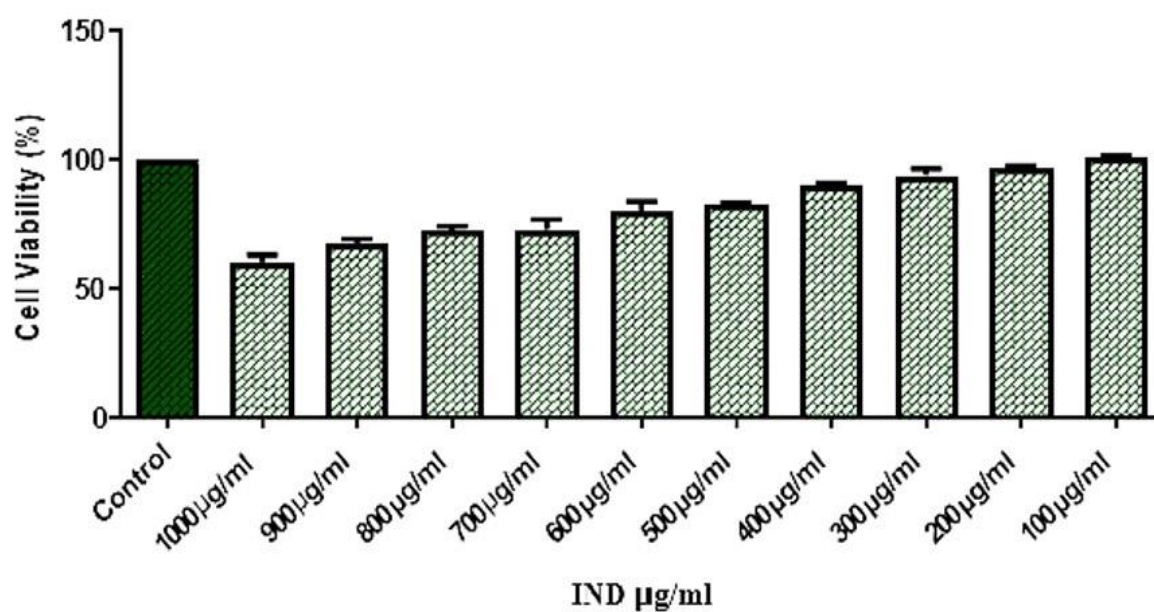


Figure 6. Cell viability of the THP-1 cells after treatment with various concentrations of immune-booster nutraceutical drink

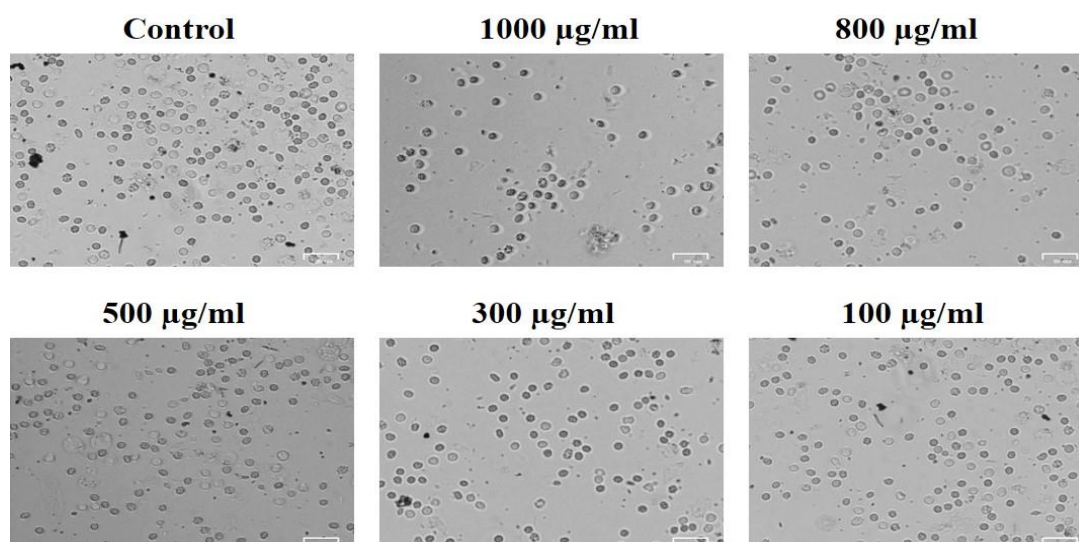


Figure 7. The MTT assay showing cell viability of THP-1 cells after treatment with various concentrations of immune-booster nutraceutical drink. Values are statistically significant ($p < 0.0001$).

Note: The THP-1 showing human monocytic leukemia cell line. Various concentrations of Immune-booster nutraceutical drink included 1000, 800, 500, 300 and 100 µg/ml.

3.4 Cytotoxicity And Enzyme-linked Immunosorbent Assay to Inhibit Interleukin 6 Using Immune-Booster Nutraceutical Drink

The MTT assay and ELISA were used to identify non-toxicity and changes in cytokine expression. The MTT assay verified non-toxicity of IND to THP-1, as a higher dose of 1000 µg/ml showed 60.05% of cell viability with an IC_{50} value of 508.1 µg ml⁻¹. The values within various concentrations were statistically significant ($p < 0.0001$; $F = 124.1$). Figures 6 and 7 show the graphical representation of the inhibition of cell viability and the microscopic view of the cells, respectively, at various concentrations. The ORSL Immunity⁺ showed no toxicity to PBMCs [18,55,62]. Thus, the non-toxic nature of IND to normal cells was verified. The IL-6 is a proinflammatory cytokine and its decrease indicates decreased inflammation. The IL-6 stimulation by lipopolysaccharide (LPS) showed increased levels of 75.62 pg ml⁻¹. After treatment, the levels decreased to 49.19 pg ml⁻¹ with an IC_{50} value of 544.5 µg/ml. This clearly indicated the decrease of IL-6 after treatment; thereby, verifying its anti-inflammatory potential and immunity development ability. The values within various concentrations were statistically significant ($p < 0.0001$; $F = 1764$). Figure 8 represents the quantified IL-6, within the control, induced IL-6 and IND treated groups. An immune combination revealed similar results, where LPS treatment showed increased expression whereas IL-6 decreased after the treatment [25,56]

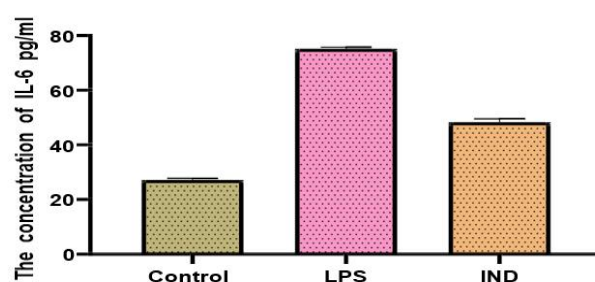


Figure 8. Quantification of interleukin-6 suppression in control, lipopolysaccharide and immune-booster nutraceutical drink-treated groups.

Note: The IL-6 refers to interleukin 6, LPS corresponds to lipopolysaccharides and IND represents Immune-booster nutraceutical drink. The values were statistically significant ($p < 0.0001$).

4. CONCLUSION

A nutraceutical drink, specifically designed to increase immunity in immunocompromised individuals, verified significant and enhanced digestion and metabolism by combining the plant components of coconut water, lemon, ginger and *Aloe vera* and supplementation of probiotic *L. acidophilus*. Accordingly, the nutritional values of IND were improved with increased carbohydrates, protein, lipid and iron and decreased glucose, which eliminated diabetic risks. The acidic pH (3.9) and decreased microbial load of (5.9×10^9 CFU ml⁻¹) verified presence of active probiotics and increased shelf life of IND. The GCMS analysis revealed the presence of 58 compounds; from which, nicotinamide demonstrated



the binding efficiency of $-3.9 \text{ kcal mole}^{-1}$ against IgE as an immunoglobulin. The non-toxic nature of IND was verified as it demonstrated low cytotoxicity to THP-1 with an IC_{50} value of $508.1 \mu\text{g/ml}$. The IL-6 as a proinflammatory cytokine was inhibited up to 49.19 pg ml^{-1} (IND treated) from 75.62 pg ml^{-1} (LPS induced) with an IC_{50} value of $544.5 \mu\text{g ml}^{-1}$. Hence, the current study verified ability of the produced IND, supplemented with probiotic (*L. acidophilus*) to possess exclusive immunity-enhancing activity by increasing the nutritional values, possessing increased shelf life, acidic pH, non-toxic nature and decreasing IL-6 cytokine.

5. DECLARATION

5.1. Acknowledgements

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5.2. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could affect the study. The authors are responsible for the content and writing of the manuscript. No conflict of interest is reported regarding the publication of this study.

5.3. Authors' Contributions

Chandran Masi: Conceptualization, project administration, methodology, investigation (formulation and probiotic supplementation), formal analysis (nutritional and shelf-life studies), writing-original draft, and visualization.

Dias Marshal P.M: Investigation (GC-MS analysis), software (molecular docking), data curation, formal analysis, and writing - review and editing.

Lijoy George: Investigation (cell culture, MTT assay, ELISA), validation, methodology, and writing-review and editing.

Miskeen Josey: Resources, supervision, validation, writing -review and editing, and final approval.

5.4. Using Artificial Intelligent Chatbots

During the preparation of this study, the authors did not use AI tools exclusively for language refinement, grammar correction and enhancing the overall clarity and readability of the manuscript. The AI tool was not used to

generate, interpret or analyze scientific data, experimental results or conclusions presented in this study.

5.5. Ethical Consideration

This study did not involve human participants or live animals. All experiments were carried out *in vitro* using established THP-1 (human leukemia monocytic) cell lines procured from a certified cell repository with food-grade plant materials and the probiotic *L. acidophilus*.

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تولید یک نوشیدنی نوتراسوتیک تقویت کننده سیستم ایمنی مبتنی بر پروبیوتیک برای افراد دچار نقص ایمنی و ارزیابی برون تنی آن

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نویسنده مسئول

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چکیده

سابقه و هدف: افراد دچار نقص ایمنی از اختلال در عملکرد سیستم ایمنی رنج می‌برند که می‌تواند منجر به التهاب، عفونت، تأخیر در بهبودی و سایر اختلالات فیزیولوژیک شود. از این رو، هدف از این مطالعه، تولید یک نوشیدنی نوتراسوتیک تقویت کننده سیستم ایمنی با استفاده از مواد اولیه گیاهی، غنی سازی آن با پروبیوتیک و بررسی ارزش تغذیه‌ای و فواید بالقوه آن برای سلامت بود.

مواد و روش ها:

نوشیدنی نوتراسوتیک تقویت کننده سیستم ایمنی با ترکیب مواد گیاهی شامل آب نارگیل سبز، زنجبیل، لیمو و آلوئه‌ورا و همچنین سایر مواد تهیه شد. سپس پروبیوتیک *لاکتوباسیلوس/سیدوفیلوس* به نوشیدنی افزوده شد و ارزش تغذیه‌ای آن، شامل میزان گلوکز، پروتئین، کربوهیدرات، چربی و آهن، تعیین شد. همچنین، به منظور ارزیابی کیفیت نوشیدنی، ماندگاری و pH آن بررسی شد. برای شناسایی ترکیبات فیتوشیمیایی موجود در نوشیدنی، از روش کروماتوگرافی گازی-طیف‌سنجی جرمی استفاده شد. برای شناسایی ترکیب دارای بیشترین پتانسیل مرتبط با تقویت ایمنی، داکینگ مولکولی ترکیبات شناسایی شده با کروماتوگرافی گازی-طیف‌سنجی جرمی در برابر Ige انجام گرفت. سمیت سلولی نوشیدنی نوتراسوتیک تقویت کننده سیستم ایمنی بر سلول‌های رده مونوسیتی لوسمی انسانی (THP-1) با استفاده از آزمون ۳-۵،۴-دی‌متیل‌تيازول-۲-یل-۵،۲-دی‌فنیل‌تترازولیم بروماید ارزیابی شد و میزان اینترلوکین-۶ با استفاده از آزمون ایمونوسوربنت متصل به آنزیم اندازه‌گیری گردید.

یافته‌ها و نتیجه‌گیری: نوشیدنی نوتراسوتیک تقویت کننده سیستم ایمنی، غنی شده با *لاکتوباسیلوس/سیدوفیلوس* (پروبیوتیک)، تهیه شد و بررسی ارزش تغذیه‌ای آن نشان داد که میزان گلوکز کاهش یافته (۰/۰۷ میلی گرم در میلی لیتر) و میزان کربوهیدرات (۸/۹۶ میلی گرم در میلی لیتر)، پروتئین (۰/۶۲ میلی گرم در میلی لیتر)، چربی (۵/۶۰ میلی گرم در میلی لیتر) و آهن (۲/۲۲ میلی گرم در میلی لیتر) افزایش یافته است. pH اسیدی ۳/۹ و کاهش بار میکروبی (۵/۹ × ۱۰^۹ واحد تشکیل دهنده کلنی در میلی لیتر) به طور همزمان فعال بودن پروبیوتیک و افزایش ماندگاری محصول را تأیید کردند. با استفاده از کروماتوگرافی گازی-طیف‌سنجی جرمی، ۵۸ ترکیب فیتوشیمیایی در نوشیدنی نوتراسوتیک تقویت کننده سیستم ایمنی شناسایی شد و نتایج داکینگ مولکولی، ویژگی‌های مرتبط با تقویت ایمنی نیکوتینامید در برابر Ige را با انرژی اتصال ۳/۹- کیلوکالری بر مول نشان داد. نتایج آزمون سمیت سلولی، غیرسمی بودن نوشیدنی را با IC50 برابر با ۵۰۸/۱ میکروگرم در میلی لیتر نشان داد و کاهش اینترلوکین-۶ نیز با IC50 برابر با ۵۴۴/۵ میکروگرم در میلی لیتر مشاهده شد. نوشیدنی نوتراسوتیک تقویت کننده سیستم ایمنی تولید شده که با پروبیوتیک غنی شده بود، از ارزش تغذیه‌ای بالاتری برخوردار بود و فاقد سمیت یا عوارض جانبی بود و می‌تواند در مطالعات آینده به منظور بررسی ظرفیت آن برای تقویت سیستم ایمنی در افراد دچار نقص ایمنی مورد بررسی بیشتر قرار گیرد.

واژگان کلیدی: *لاکتوباسیلوس/سیدوفیلوس*، تغذیه، سمیت سلولی، ماندگاری، داکینگ مولکولی.

