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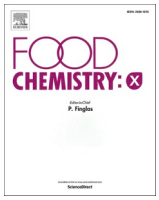

Characterization of jamun (*Syzygium cumini*) juice fortified with nanoemulsified vitamin D_3: *In vitro* and *in vivo* assessment of its nutraceutical value and anti-diabetic potential

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A B S T R A C T

This study aimed to fortify Jamun (*Syzygium cumini*) juice with vitamin D_3 to address vitamin D deficiency and boost health. A nanoemulsion of vitamin D_3 was fabricated using a low-temperature (4–20^{0}C) sonication method and incorporated into the juice. The vitamin D fortified jamun juice (VDFJJ) exhibited a total polyphenol content of 14.37 mg GAE/mL, total flavonoids of 8.27 mg QE/mL, and 94.2 % antioxidant activity. It demonstrated antidiabetic potential, with IC_{50} values for α-amylase and α-glucosidase inhibition at 110 μg/mL and 134 μg/mL, respectively. Vitamin D_3 showed 82 % release profile in simulated gastrointestinal fluids. After 4 weeks of VDFJJ intervention in vitamin D-deficient animal models, serum levels of 25-OHD, PTH, calcium, phosphorus, and ALP were significantly improved. Vitamin D_3 demonstrated stability within the matrix, showing a slight reduction from 4000 IU to 2440 IU over a three-month period. This nanoemulsion approach effectively enhances the solubility and bioavailability of vitamin D_3 in low-fat beverages like jamun juice, offering significant nutritional benefits and anti-diabetic properties.

1. Introduction

Vitamin D is a fat-soluble vitamin, which is synthesized by the body when its precursor 7-dehydrocholesterol is exposed to sunlight. The inactive form of vitamin D gets converted to its active form by the first hydroxylation in the liver forming 25-hydroxyvitamin D (calcidiol) followed by the second hydroxylation in the kidney forming 1, 25-dihydroxyvitamin D (calcitriol) (Ramasamy, 2020). The biological functions of vitamin D are related mainly to bone formation, osteoporosis, and calcium homeostasis. It also plays a key role in other non-communicable diseases like diabetes, cardiovascular diseases, hypertension, cancer, and other autoimmune diseases (Jan et al., 2019). Notably, vitamin D deficiency persists globally and about 1 billion people are estimated to be vitamin D deficient in regions including India, Australia, Europe, America, and the Middle East (Malik, Jan, Haq, et al., 2022). Vitamin D deficiency can result from avoiding sun exposure, excessive use of

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sunscreen, or cultural practices involving clothing that covers most of the skin (Carlberg, 2016). The natural dietary sources of vitamin D are very limited, such as fatty fish, egg yolks and cod liver oil, are not consumed widely enough to address deficiencies in the general population. Coupled with reduced sun exposure due to lifestyle factors, novel strategies like fortification in foods have become crucial to overcome vitamin D insufficiency. In recent years food fortification strategies have been adopted in some countries to overcome and prevent vitamin D deficiency.

The countries like US and Canada have been undertaking the fortification of food, especially milk products. However, fortifying a single staple food (milk) with vitamin D may not satisfy the needs of those consumers who have lactose intolerance or allergies (Cashman & Kiely, 2016). Thus, additional strategies for the fortification of a wider range of potential foods need to be considered to increase intakes of vitamin D.

On the other hand, jamun (*Syzygium cuminii*) is one of the important, yet underutilized indigenous crops of India. The jamun fruit is rich in antioxidants, phenols, and anthocyanins which not only help in maintaining a good and healthy lifestyle but also in combating various diseases like cardiovascular, hypertension, and diabetes in particular. Diabetes is the most prevalent endocrinological disorder in the world (Paul & Das, 2018). The total number of people with diabetes is estimated to increase to 643 million by 2030 worldwide and is expected to increase to 783 million by 2045 (International Diabetes Federation, 2021). Jamun also possesses other medicinal properties like anti-cancer, anti-fungal, anti-bacterial, and anti-diabetic (Rehaman, 2021). The whole contour of the jamun tree including its bark, leaves, pulp, and seeds have medicinal properties (Suradkar et al., 2017). The bioactive compounds like polyphenols and carotenoids are reported to have a beneficial effect on health and the extracts of jamun are used to cure cough, cold, asthma and obesity (Rauf et al., 2021).

The literature indicates that jamun exhibits antiviral properties and suggests that consuming antioxidant-rich foods can enhance immunity and help in regulating blood glucose levels (Singh & Gupta, 2007). More interestingly the role of vitamin D and jamun has been reported to boost immunity during the Covid-19 pandemic (Grant et al., 2020; Rehaman, 2021). Another study demonstrates that vitamin D supplementation in adults significantly reduced the risk of type 2 diabetes (Barbarawi et al., 2020).

To meet the forthcoming demands of society it becomes necessary for the food sectors to enhance the functional value of various underutilized crops. For developing a value-added fortified beverage with a homogeneous distribution of bioactive compound (vitamin D_3) with improved stability and bioavailability, a nanoemulsion-based technique was employed. Based on the individual properties that enhance the overall stability, solubility, and effectiveness of the nanoemulsion, **Medium-chain triglycerides (MCT) oil** was chosen as the carrier due to its ability to improve absorption and provide stability to the formulation. **Kolliphor RH-40 (KRH-40)** was selected as the surfactant for its excellent emulsifying capabilities, which facilitate the stabilization and solubilization of vitamin D_3. Additionally, **Ethylene Glycol (EG)** was used as a co-surfactant to improve the formulation's stability and enhancing the overall performance of the nanoemulsion (Jan et al., 2022). Jamun possesses huge potential for the processing and development of a suitable functional food product with a range of health benefits (Jan et al., 2021). Thus, keeping in view the vitamin D deficiency and health benefits of phytonutrient-rich jamun. Fortification of jamun juice with the pre-emulsified vitamin D_3 was done to achieve a fortification level of vitamin D_3 up to 4000 IU per serving. Such functional food beverages besides providing basic nutrients will offer several health benefits. This study aimed to explore the fortification of jamun juice with vitamin D_3 as a dual-purpose intervention to combat vitamin D deficiency and curtail the burden of diabetes.

2. Material and methods

2.1. Materials

The freshly harvested berry (jamun) was obtained locally from a fruit market, located in Okhla, New Delhi, India. Vitamin D_3 standard was purchased from Sigma Aldrich (India). HPLC grade solvents (acetonitrile, and methanol), DPPH (2, 2 diphenyl-1-picrylhydrazyl), Folin-Ciocalteu's reagent were purchased from Merck (India).The additional chemicals/ reagents (analytical grade) used in the experiments were purchased from SD Fine Chemicals, India.

2.2. Extraction of juice from jamun fruit

The procured jamun fruit, free from cuts or damage, was washed, cleaned, and de-seeded manually. Juice extraction was carried out using a **laboratory blender (**Philips HR3752) **that** took approximately **5–10** min. The extracted juice was then filtered for clarification using a muslin cloth to remove pulp and other solids. Finally, the juice underwent pasteurization at **75–80 °C for 15 min** to eliminate microbial contaminants. The juice was promptly cooled to 25 °C, transferred into sterilized polyethylene terephthalate (PET) bottles, and stored for further study.

2.3. Yield determination

2.3.1. Consumable matter (%)

Fully ripe jamun fruit was weighed and deseeded manually. The weight of the deseeded fruit was measured. The utilizable matter in terms of percentage was calculated as below.

$$\%Utilizable\ matter = Wt\ of\ deseededfruit(g)/Wt.of\ whole\ fruit(g) \times 100 \quad (1)$$

2.3.2. Recovery of juice from jamun pulp

The utilizable portion was weighed, and extraction of the liquid portion was done using a blender (Nissan 300 W). The clear liquid was collected by passing the mashed pulp through a muslin cloth separating the solid residue. To determine the juice recovery percentage, the clear juice was transferred into a measuring cylinder and the percentage was calculated as:

$$\%Juice\ Recovered = Vol.of\ strainedjuice\ (ml)/wtof\ fruit\ (g) \times 100 \quad (2)$$

2.4. Proximate analysis of jamun juice

The proximate analysis of jamun juice was done by following methods given by the Association of Analytical Chemists. All the experiments were conducted in triplicates and results were expressed as mean ± standard deviation.

2.4.1. Moisture content

For the determination of the water content, 5 mL of the juice were taken in pre-weighed petriplates and kept in a hot chamber (time of 24 h, temperature of 110 °C). The difference in weights before and after drying was expressed in terms of moisture percentage.

2.4.2. Crude protein

The Kjeldahl method was followed for the evaluation of crude protein of extracted liquid. The protein determination was carried out in various stages. The digestion was carried out by mixing of 10 mL sample with 10 mL of concentrated sulfuric acid. Further potassium sulfate and copper sulfate were added in a ratio of 10:1. The reaction mixture in the digestion phase continued at temperatures ranging from 350 to 380 °C for about 1 h. The holding tubes containing the sample were cooled to ambient temperature at the end of the digestion process. The holding

tubes containing the sample were then subjected to distillation, and ammonia gas was formed from ammonium ions with the addition of alkali (40 % NaOH). The gas formed was trapped in a boric acid solution (4 %). In the last phase, titration was carried out against 0.1 N hydrochloric acid solution using methyl red and bromocresol green as indicators to determine the total nitrogen in the condensate flask. A nitrogen conversion factor of 6.25 was used to estimate protein.

2.4.3. Crude fat content

Fat content was determined using the soxhlet apparatus (SocsPlus Equipments, India) following the solvent extraction method.

2.4.4. Crude fiber content

The crude fiber content was determined using the acid and the alkali digestion method as per protocol.

2.4.5. Ash content

To determine the ash content in the samples, 5 mL of the juice was poured into a high-temperature resistant silica crucible and kept in a muffle heating system for 3 h at 400 °C. The ash containing silica dishes were cooled down and weight was recorded for the estimation of ash.

2.4.6. Total carbohydrates

The content of carbohydrates was measured by difference to 100 % of major constituents (Fiber, ash, protein, and fat).

2.4.7. Total sugar

The total sugars were estimated by the phenol–sulfuric acid method.

2.4.8. Vitamin C

Estimation of vitamin C was made using the 2, 6-dichloroindophenol titration method.

2.4.9. Pectin content

For determination of the pectin content, a method by Ghosh et al. (2017) was followed. Near about 5 mL of juice, the sample was treated with 50 mL of ethanol followed by thorough mixing and boiling for 30 min. The mixture was then filtered using Whatman filter paper separating the residue and filtrate. The whole process was repeated till the alcohol solution turned colorless. The alcohol-insoluble solids left on the filter paper were dried and the residue was kept in a hot air oven at 100 °C for 1–2 h. After cooling pectin content was calculated as (*W*/W %)

$$Pectin\% = wt\ of\ dried\ residue(g)/wtof\ sample\ taken\ (g) \times 100 \quad (3)$$

2.4.10. Viscosity measurement

The viscosity of the formulated beverage was determined using a brook filed digital viscometer (Scientific Instruments MRC VIS-S2, India) at room temperature (25 ± 5 °C). The unit of measurement used for viscosity was mPa.s.

2.5. Analysis of total phenol content (TPC), total flavonoid content (TFC), and antioxidant activity

The total phenols, flavonoids, and DPPH antioxidant activity of the fortified jamun juice were determined by methods demonstrated by Malik et al. (2019).

For the study, acidified methanol was used to extract 20 mL of sample juice and centrifugation was done for 10 min at 5000 rpm. The centrifuged material was dried by rota-evaporator and estimation of TPC, TFC and antioxidant potential was calculated. All the samples were evaluated in triplicates.

The TPC in the jamun juices sample was estimated using Folin–Ciocalteu's reagent. Briefly to 100 µL of the Folin–Ciocalteu reagent, 20 µL of jamun juice sample, and 1.58 mL water was added, followed by the addition of 300 µL of the (20 % *w/v*) sodium carbonate solution. The solution was incubated at 20 °C for 2 h. The absorbance was measured by spectrophotometer (SpectroScan 80DV), at 765 nm and the results were expressed as mg gallic acid equivalents (GAE)/L.

For the estimation of TFC in jamun juice, one mL of the methanolic extract, 300 µL of 5 % $NaNO_2$ was added, followed by 600 µL of $AlCl_3$ (10 %). Further to the reaction mixture 2 mL of NaOH (4.3 %) solution was added, and the absorbance was recorded at 510 nm, and the results obtained were expressed in terms of mg Quercetin (QE)/L.

DPPH (2, 2- diphenyl-1-picrylhydrazyl) radical scavenging method was used to estimate the antioxidant activities of the jamun juice. To100 µL of methanolic extract, 290 µL of DPPH (0.1 mM) solution was added and thoroughly mixed. The mixture was kept in the dark for 1 h and the reduction in absorbance was recorded at 517 nm with the help of a spectrophotometer (SpectroScan 80DV). The results of the DPPH antioxidant assay was expressed in terms IC_{50} values.

2.6. Preparation of emulsion for fortification of the jamun juice with vitamin D_3

The technique of formulating a nanoemulsion derived from our previous study (Jan et al., 2022) which employs a sonication method at low temperatures to add vitamin D_3 into the oil water emulsion to develop a surfactant-based colloidal delivery system. The process of creating an emulsion system involved blending of medium chain triglyceride (MCT) as a carrier oil for vitamin D_3, Kolliphor-RH-40 as a surfactant, ethylene glycol as a co-surfactant, and deionized water. Further particle size and zeta potential of formulated emulsion were examined by employing photon correlation spectroscopy (Nano = ZhniquS-90, Malvern, UK). The mean ± SD was calculated by performing each sample measurement in triplicates. Before the fortification, jamun juice was standardized by using various food-grade stabilizers (sodium alginate, alginic acid, ascorbic acid,) and preservatives.

2.7. Preparation of juice for fortification

To prepare fortified juice, 0.02 % citric acid was used as an acidity regulator, chelating agent, and colour enhancer, while 0.8 % sodium alginate served as an emulsifier in the final formulation. Sodium benzoate (100 ppm) was incorporated as a preservative to inhibit yeast and mold growth, thereby extending the product's shelf life. Before fortification, the jamun juice was optimized using food-grade stabilizers such as sodium alginate, alginic acid, and ascorbic acid. Emulsion containing vitamin D_3 was directly added to the juice at a concentration of 20 IU/mL and subsequently homogenized with a magnetic stirrer for 10 min to ensure uniform distribution. Stabilization of the juice was achieved through low-temperature storage. The fortified jamun juice (200 mL) containing vitamin D_3 (4000 IU) was packed in PET containers, and stored under refrigeration for further analysis.

2.8. Physicochemical characterization of fortified beverage

2.8.1. Fourier transformation infrared spectroscopy

The FTIR spectra of vitamin D-fortified jamun juice (VDFJJ) were obtained by using FTIR spectrometer (IR Affinity SHIMADZU, Japan) instrument. Samples were mixed with dehydrated crystalline potassium bromide (KBr) and pellets were prepared. This mixture was placed into a fine powder by agate mortar before being pressed into the KBr disc. The KBr discs were evaluated in the wave-number area 4000–400 cm^{-1}.

2.8.2. Differential scanning calorimetry

The thermal stability of VDFJJ was done by DSC (Pyres DSC 6, Perkin Elmer USA).VDFJJ was freeze-dried. Heat treatment with a temperature range of 40 to 350 °C at 10 °C/min under 20 mL/min supply of nitrogen was given to the required amount of sample (1–2 mg).

2.9. Stability studies of fortified jamun juice during storage

The stability study of the optimized beverage was conducted for three months at 15-day intervals and the parameters analyzed were total soluble solids (TSS), pH, titrable acidity, anthocyanin content, vitamin D content, colour profile, and microbial study (Total plate count, Total yeast count).

2.9.1. Determination of total soluble solids

Digital Abbe refractometer (MRC K7135) was used to determine the total soluble sugar (TSS) and expressed as degree Brix (°B).

2.9.2. Determination of pH and titrable acidity

The pH of jamun juice was estimated with the help of a pH meter (Toshcon Pvt. Ltd. India). Titrable acidity was measured by titrating with 0.2 N NaOH using phenolphthalein as a marker and the result was given as the percentage of citric acid.

2.9.3. Anthocyanin content

For the estimation of total anthocyanins, the pH differential method was adopted. Using 0.025 M of potassium chloride and 0.4 M of sodium acetate, aqueous solutions at pH 1 and 4.5 were made, pH adjustment was done with concentrated HCl. To the 200 µL of the extracted sample, 1.8 mL of buffer solution was added. The mixture was then kept in the dark for about 15 min the absorbance at 510, and 700 nm was recorded using UV–visible spectrophotometer (Spectro Scan 80DV) using deionized water as the blank, and the results were calculated using the following formula:

$$\text{mg cyd} - 3 - \text{glu mL}^{-1} = (Abs \times \text{DF} \times \text{MW} \times 1000)/(\varepsilon \times \text{L}) \quad (4)$$

where *Abs* is the (pH 1: Abs_{510} nm − Abs_{700} nm) − (pH 4.5: Abs_{510} nm − Abs_{700} nm), MW is the molecular weight, DF is the dilution factor, L is the path length (1 cm) and ε is the molar extinction coefficient. For cyanidin-3-glucoside (cyd-3-glu), the molecular weight (449.38 g mol^{-1}) and molar extinction coefficient (26,900 M^{-1} cm^{-1}) were used.

2.9.4. Determination of vitamin D_3 in VDFJJ

The high-pressure liquid chromatography (HPLC) technique as described by Dimartino (2007) with minor modification was used to quantify vitamin D_3 in the fortified beverage. Near about 10 mL of sample was taken in a vial and an equal amount of hexane was added. The mixture was thoroughly mixed and ultrasonicated for 10 min at room temperature. Centrifuging the mixture for 6 min at 6000 rpm was followed by the parting of the transparent hexane layer, which was rotary-evaporated until it was completely dry. Around 20 µL was loaded to the HPLC system (Waters, USA), which had both a Waters 996 photodiode array detector and the Li-Chromosphere RP-C18 column (125–5 mm). In this method, an 80:20 ratio of methanol and acetonitrile was used as the mobile phase with a flow rate of 1 mL/min. Detection was carried out at a wavelength of 264. Vitamin D_3 was identified by comparing the retention time with the standard. The area under the curve was used to calculate the vitamin D_3 content in the emulsion.

2.9.5. Colour measurement

The Colour measurements were performed by a handy spectrophotometer, Lovibond LC 100 (X-Rite, Incorporated). To calibrate the instrument before taking the measurements, we used plate standard with D_{65} illumination. The colour parameters (L, a, b, h and C) were depicted as brightness/darkness "L", "a" is redness/greenness, "b" is yellowness/blueness, "h" is Hue, "C" is chroma and overall colour variation "dE" were expressed using eq. 5, 6 and 7 respectively:

$$\text{"h"} = 1/tan\,(\text{b/a}) \quad (5)$$

$$\text{"C"} = \sqrt{(\text{a}^2 + \text{b}^2)} \quad (6)$$

$$\text{"dE"} = \sqrt{(L - \text{L}_0)^2 + (\text{a} - \text{a}_0)^2 + (\text{b} - \text{b}_0)^2} \quad (7)$$

2.9.6. Microbiological studies

Bacteria, yeast, and mold count were determined as described by del Socorro Cruz-Cansino et al. (2015) with slight modifications. To enumerate the total bacterial count, the VDFJJ samples were diluted serially (10-fold) in peptone water (0.1 %), and for the yeasts and mold viable counts VDFJJ samples were serially diluted in distilled water. These were further plated out on nutrient agar media for total plate count (TPC) and on potato dextrose agar media for yeasts and mold count. Plates were incubated at 30 °C for 48 h for TPC and 25 °C for 72 h for yeast and mold count. Colonies were enumerated, and the number was recorded as colony-forming units (CFU) per mL. The samples were analyzed in triplicates.

2.10. In vitro assays

2.10.1. Determination of alpha-amylase activity

For the determination of the inhibitory alpha-amylase activity of the developed fortified jamun juice, 100 µL of the sample was mixed with 100 µL sodium phosphate buffer- 0.02 mol/L (pH 6.9) and α-amylase solution 10 µL. The reaction mixture after 10 min was mixed with 100 µL of starch solution (1 %) and was allowed to incubate for 30 min at 25 °C. Finally, the reaction was ended by adding 1 mL of dinitro salicylic acid reagent. For pre-warming, the tubes containing reaction mixtures were placed in a water bath (100 °C) for 5 min. The reaction mixture tubes were then cooled to 25 °C and diluted ten-fold with deionized water. The absorbance at 540 nm was recorded (Sathiavelu et al., 2013).

2.10.2. Determination of glucosidase activity

The α- glucosidase activity was determined by a method as reported by Rauf et al. (2021). 100 µL of the sample was mixed with phosphate buffer 0.1 M (pH 6.7) and 100 µL of α- glucosidase enzyme followed by the incubation for 15 min at 35 °C. Further *p*-nitrophenyl-α-D-glucopyranoside (5 mM) was added to the reaction mixture, mixed well, and again incubated at 30 °C for 15 min. Finally, 1 N Na_2CO_3 (1 mL) was added, and with the change in colour to yellow, absorbance was recorded at 400 nm using a spectrophotometer. The value of α-glucosidase assay was expressed in IC_{50} value.

2.10.3. In-vitro release of vitamin D_3 in gastric and intestinal fluids

Simulated gastric (pH 1.2) and intestinal fluids (pH 7.4) were used to determine vitamin D_3 release profiles from VDFJJ by dialysis bag diffusion method (Molaveisi et al., 2020). The dialysis membrane was obtained from Sigma Aldrich India, with a molecular weight of 12 k Dalton, 5 mL of the VDFJJ containing about 2000 IU of vitamin D was placed in a dialysis sac and was closed at two ends. For 2 h, the dialysis sac was kept in a gastric simulated solution (pH 1.2, 90 mL) with a constant moving state (100 rpm) and then for 4 h at 37 ± 0.5 °C were inserted in intestinal simulated fluid (pH 7.4, 90 mL). At 0, 30, 60, 90, 120, 240, 360 and 480 mins, the samples were withdrawn and analyzed using HPLC at 264 nm. The percentage of vitamin D released cumulatively was calculated.

The release data was utilized for kinetic modeling, which involved zero order, first order, Higuchi, Korsmeyer-Peppas, Pappas Sahlin, Hopfenberg, and Quadratic models (Eqs. 8–14) to determine the potential release of vitamin D from the juice matrix.

$$\text{C} = \text{kt} \quad (8)$$

$$\text{C} = 100[1 - \text{Exp}\,(-\text{kt})\,] \quad (9)$$

$$\text{C} = \text{kt}^{0.5} \quad (10)$$

$$\text{C} = \text{kt}^{\text{n}} \quad (11)$$

$$C = k_1 t^{0.5} + k_2 t \quad (12)$$

$$C = 100 \times [1 - (1 - kt)^n] \quad (13)$$

$$C = 100 \times (k_1 t^2 + k_2 t) \quad (14)$$

The concentration of vitamin D_3 at time t is referred to as "C", the kinetic constant is referred to as "k" and the release exponent is referred to as "n"

2.11. Bioavailability of vitamin D in animal models from VDFJJ

To assess the bioavailability of vitamin D_3 from VDFJJ, 24 Wistar albino rats were selected due to their physiological similarity to humans in vitamin D metabolism and diabetes, making them an appropriate model for this study. The rats, aged 70–90 days with an average body weight of 150 ± 30 g, were procured from the Central Animal House Facility (CAHF), Jamia Hamdard, New Delhi, India. After approval from the Institutional Animal Ethics Committee, Jamia Hamdard (Registration no. 173/GO/Re/S/2000/CPCSEA, proposal no 1556/2019), the study was carried out as per the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Govt. of India. The bioavailability of vitamin D_3 from the VDFJJ was done by the incorporation of the VDFJJ in vitamin D deficient animals. The animals were supplied with the vitamin D deficient diet (based on AIN-93G formulation, for three weeks Bio-Serve, NJ, USA) with ad libtum water supply and were devoid of exposure to tube or solar light. The confirmation of vitamin D deficiency in animal models was confirmed from the serum levels of 25OHD levels by CLIA method upon dosage with a vitamin D deficient diet for four weeks. Animal models with <25 ng/mL of the serum levels of serum vitamin D were considered as vitamin D deficient. The experimental group was further subdivided as G-2; G-3; G-4 consisting of 6 representatives as described:

G-1: Fed on a vitamin D deficient diet (Control Group).

G-2: Fed on a vitamin D deficient diet + plain jamun juice.

G-3: Fed on a vitamin D deficient diet + VDFJJ.

G-4: Fed on a vitamin D deficient diet + vitamin D_3 supplement (Calcirol, Cadila Pharmaceuticals Ltd., India).

2.12. Blood sampling and analysis of biochemical parameters

For biochemical examination, blood samples were taken through the retro-orbital plexus under gentle anesthesia (ether). Following the 28-day study, the cardiac puncture was done to collect blood (2-3 mL/rat) from the rats while they were under deep anesthesia (3–5 % isoflurane) in a vaporization compartment and quickly cervical dislocation was carried out as per ethical norms. Blood samples were collected in yellow-toped tubes (Nexamo Vacutainer, Mohali, India) and then centrifuged for 10 min at approximately 1400 ×*g* or 3000 rpm. The parameters which were analyzed were as: Serum 25(OH) D, Serum Calcium, Serum phosphorous, PTH and alkaline phosphate (ALP) using kit method (Malik, Jan, Al-Keridis, et al., 2022). The separated serum was collected and kept at −80 °C till analysis time. Repetitive defrosting and freezing of serums was avoided.

2.13. Serum 25-hydroxy vitamin D

The Advia Centaur assay (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) is used for the quantification of total vitamin D, utilizing a releasing reagent and a monoclonal antibody to detect 25-hydroxyvitamin D_3 (25-OH D_3) (Malik, Jan, Haq, et al., 2022).

2.14. Estimation of parathyroid hormone levels (PTH)

For PTH measurement Advia Centaur (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) intact PTH assay (monoclonal antibodies assay) was used by following a standard protocol (Risteli et al., 2015).

2.15. Serum calcium, phosphorous and alkaline phosphatase

The assessment was done by semi-auto-analyzer Erba Chem-5 (Erba Diagnostics, Manheim, Germany) *via* market-accessible kits by following the maker's protocol (Malik, Jan, Al-Keridis, et al., 2022).

2.16. Sensory analysis

To assess the suitability of fortified VDFJJ, sensory analysis was done on the hedonic scale of 1–9. Various sensory attributes (taste, colour, flavor, tartness, appearance, and overall acceptance) were evaluated by the trained panelists for sensory assessment of VDFJJ.

2.17. Statistical analysis

All experiments were conducted in triplicates and data were expressed as mean ± SD. Statistical analysis of experimental data was performed by analysis of variance (ANOVA). The graphs were plotted using GraphPad Prism 8.

3. Results and discussion

3.1. Consumable and juice recovery

The total consumable matter of the jamun fruit was around 80.4 % and the inedible part (seeds and residue) was 19.6 %. The percentage of the juice recovered from the consumable edible part was around 56.7 % (Fig. 1). Our findings were in line with (Ghosh et al., 2017) who reported 54 % of the juice, 79 % of consumable parts, and 19 % of non-consumable parts from the jamun. In another study by (Suradkar et al., 2017), the percentage of juice recovery was around 53.7 % of consumable matter around 77.53 %, and 22.4 % of inedible matter.

3.2. Proximate analysis

Table 1 summarizes the proximate composition of jamun juice. The analysis revealed the moisture content (83.2 %), crude protein (0.92 %), fat (0.45 %), fiber (0.76 %), ash (0.98 %), carbohydrates (13.69 %), total soluble solids (10.5^0 Brix), pectin (6.5 %), viscosity (1.41 ± 0.05 Pa.S), ascorbic acid (15.3 mg/100 mL),Total sugar (11.6 %) reducing sugars (9.68 %) and non-reducing sugar (2.08 %), for jamun juice. In comparison Ghosh et al. (2017) have reported slightly different value for moisture content of (79.21 %), ash (1.03 %), crude protein (0.65 %), Fiber (0.53), fat (0.18), TSS (14.86), total solids (20.33), pectin (4.7), viscosity (1.03 PaS), and reducing sugars (7 %). These differences may be attributed to variations in raw material or processing methods. The rich nutritional profile of jamun highlight its potential health benefits, supported by similar findings by Shahnawaz et al. (2009) in studies on jamun jam preparation.

3.3. Total phenolic content of VDFJJ

The phenol content in the VDFJJ was found to be 14.37 ± 1.13 mg GAE/mL. These results align with our previous findings (Jan et al., 2021) where we obtained 7.89 mg GAE/mL in the whey-based jamun ice popsicle formulation containing 50 % jamun juice combined with whey. Some previous studies have also reported similar findings like, 11.41 mg GAE/g in jamun pulp (Shahnawaz et al., 2009), 11.17 mg GAE/mL in fresh jamun juice (Rufino et al., 2011), and 21.33 mg GAE/g in jamun (Suradkar et al., 2017). The total phenolic content in jamun juice primarily comprises flavonoids, tannins and anthocyanins. The concentration of phenolic compounds in the juice can vary depending on factors such as ripeness, extraction methods, and environmental

Fig. 1. Processing of VDFJJ; jamun fruit (a), jamun juice (b), seed and residue (c), vitamin D_3 nanoemulsion (d).

Table 1
Proximate analysis and physiochemical composition of jamun juice.

Parameters	Values
Moisture (% wet basis)	83.2 ± 1.23
Crude protein (%)	0.92 ± 0.07
Fat (%)	0.45 ± 0.03
Fiber (%)	0.76 ± 0.05
Ash (%)	0.98 ± 0.05
Carbohydrates (%)	13.69 ± 0.93
Total Sugar (%)	11.76 ± 1.2
Reducing Sugar (%)	9.68 ± 0.68
Non-Reducing Sugar (%)	2.08 ± 0.23
Ascorbic Acid (mg/100 mL)	15.3 ± 1.12
pH	4.2 ± 0.2
Acidity (% citric acid)	0.32 ± 0.02
Viscosity (Pa.s)	1.41 ± 0.05
Total Soluble Solids (^{0} Brix)	10.5 ± 1.05
Total Polyphenols (mg GAE/mL)	14.37 ± 1.13
Total Flavonoids (mg QE/mL)	8.27 ± 1.03
Anthocyanin (mg/mL)	5.5 ± 1.73
DPPH Antioxidant Activity (IC50 value)	53.37 ± 0.02

The data is expressed as mean values of triplicates ± SD.

conditions (Patras et al., 2010).

3.4. Total flavonoid content of VDFJJ

Total flavonoids were found to be 8.27 ± 1.03 mg QE/mL, our findings were found to be in line with the earlier study by Jan et al. (2021) where they reported 4.58 mg QE/mL of flavonoids in the whey jamun ice popsicles using 50 % of jamun juice, other research conducted by (Sundararajan et al., 2016), reported 5.34 mg QE/mL of flavonoid content in jamun squash (Mohamed et al., 2013) reported 6.22 mg QE/g in methanolic extract of dried jamun sample. The bioactive compounds including flavonoids in jamun juice are known for their role in neutralizing free radicals and supporting health.

3.5. DPPH antioxidant activity of VDFJJ

The findings for the DPPH assay were expressed in terms of IC_{50} values, which indicated that the lower value of IC_{50} indicates enhanced antioxidant activity. Maximum DPPH antioxidant activity (% inhibition) of methanolic extract of jamun juice was found to be 94.2 ± 2.6 %. The IC_{50} value of jamun juice was 53.37 ± 0.02 μg/mL. The IC_{50} value of ethanolic extract of jamun was reported as 56.92 μg/mL by Patel et al. (2019). Moreover, a recent study by Ahmed et al. (2021) reported a higher IC_{50} value of 281 ± 1.31 μg/mL for jamun pulp, however the findings for jamun seeds were 11.78 ± 0.08 μg/mL. In addition, a study conducted by Qamar et al. (2022) demonstrated that the methanol fruit extract of *S. cumini* exhibited a strong antioxidant property in the DPPH (81.4 μg/mL) assay. Jamun juice demonstrates significant DPPH radical scavenging activity, indicating its potent antioxidant capacity. The level of antioxidant activity can be influenced by factors such as the juice's phenolic content.

3.6. Physicochemical characterization

3.6.1. Particle size and zeta potential of the vitamin D_3 emulsion

The formulated nanoemulsion was determined to have a particle size of 169 ± 1.32 nm and the zeta potential was −26 mV (Fig. 2).The optimum droplet size for the nanoemulsions ranges from 20 to 200 nm (Solans et al., 2005) the potential value of more than +25 mV and less than −25 mV reflect higher stability (Samson et al., 2016). Further, it is important to predict the long-term stability of formulated nanoemulsion, zeta vitamin-D-rich nanoemulsion (Fig. 1d) was incorporated into jamun juice for application as food fortificant.

3.6.2. FTIR analysis

The VDFJJ was analyzed using Fourier transformation infrared spectroscopy (FTIR).All the components used in the nanoemulsion formation *viz.*, pure vitamin D_3, blank jamun juice (vitamin D_3 free), and VDFJJ were conducted to observe any probable chemical interface connecting vitamin D_3 and other components of the beverage. The IR spectra of vitamin D_3 containing beverage and blank are represented in

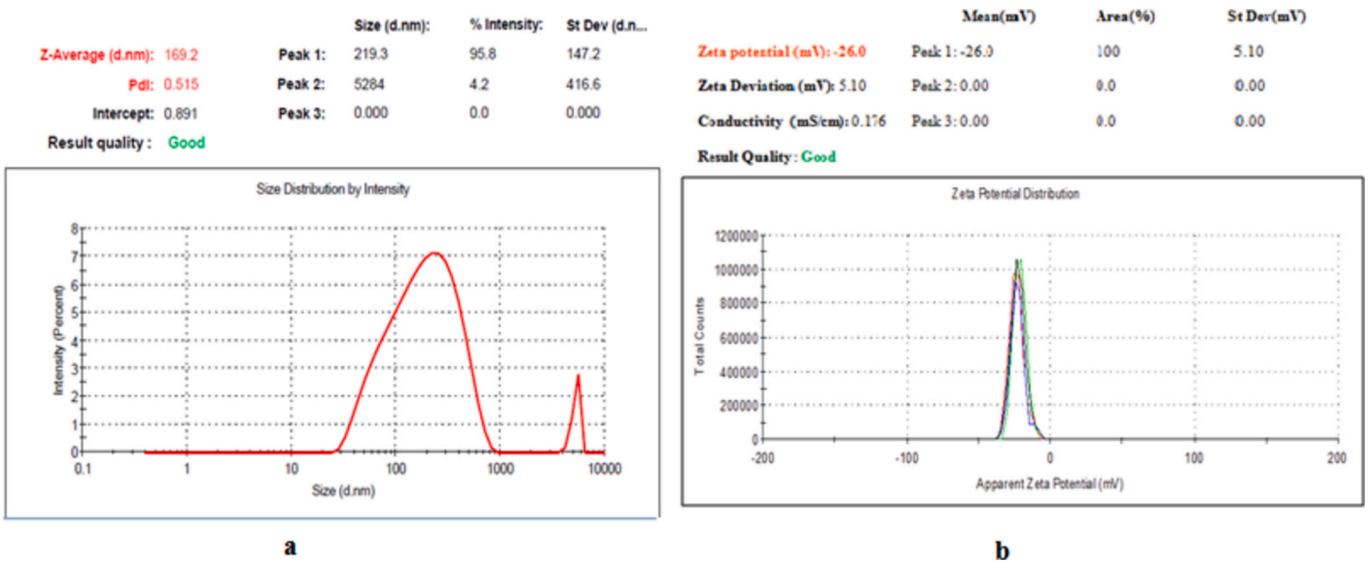

Fig. 2. Size distribution (a) and zeta potential (b) of nanoemulsion.

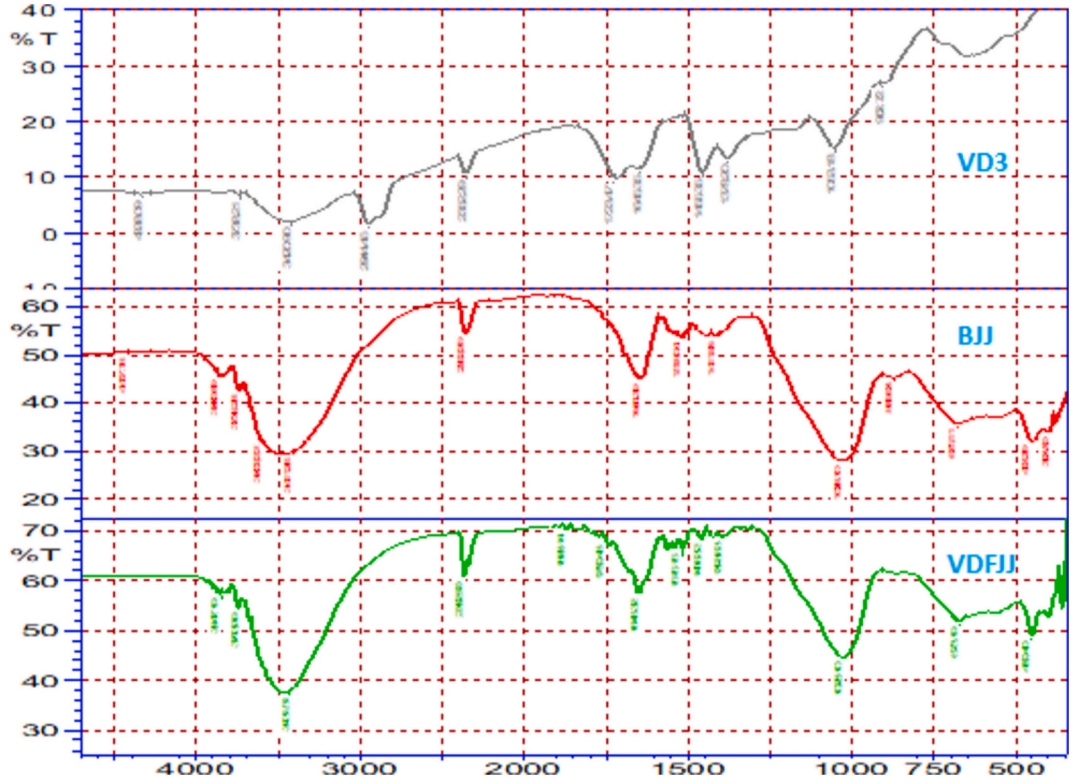

Fig. 3. FTIR spectra of vitamin D_3 (VD3), blank jamun juice (BJJ) and vitamin D_3 fortified jamun juice (VDFJJ).

Fig. 3. In the vitamin D_3 spectrogram, a series of bands were found particularly C═O stretching aldehyde (1723 cm^{-1}), C—O group (2352 cm^{-1}), alkyl C—H stretches (2944 cm^{-1} and 2853 cm^{-1}), hydrogen bond O—H stretching (3420 cm^{-1}), and various other peaks in the wave number range of 1645–900 cm^{-1} indicating C—H bending (Thoke et al., 2013). In the spectrum of blank beverage containing jamun juice only, bands at 3860 cm^{-1}- 3736 cm^{-1} represent O—H stretching vibration, 3602 represent amide bonds, 3411 cm^{-1} represent the hydrogen bond O—H stretching (Sharma et al., 2017), C—O group 2352 cm^{-1}, 1518 cm^{-1}, 1411 cm^{-1} indicating C═C stretch of amines and alkenes aromatic stretch, 869 cm^{-1} indicates C—H bonding of phenyl ring, 675 cm^{-1} represents C—H bond of alkynes and 453 cm^{-1} represents C—H bonding of alkyl halide functional group. In the spectrum of the fortified beverage presence of both the jamun peaks and vitamin D_3 was seen. Series of bands were observed like C—O group (2362 cm^{-1}), C═O stretching aldehyde (1750 cm^{-1}), and several peaks in the wave number range of 1645–900 cm^{-1} indicating C—H bending. Peaks in the wave number range from 3847 cm^{-1} to 3743 cm^{-1} indicate the weak O—H stretching vibration. The presence of the vitamin D_3 peaks in the fortified jamun juice indicates that there is no compound interfaces between vitamin D_3 and the phytochemical component of jamun juice. Similar peaks of vitamin D_3 were also observed by Maurya and Aggarwal (2019)

and Molaveisi et al. (2020) in their studies of encapsulating vitamin D.

3.6.3. DSC analysis

DSC was conducted over a temperature range of 40 °C to 350 °C to assess thermal property modifications, including melting temperature, glass transition, and crystallization behavior of the pre-emulsified vitamin D_3 and jamun juice mixture. The DSC profiles for vitamin D_3, the blank beverage, and the vitamin D_3-fortified beverage were analyzed. A distinct melting peak for pure vitamin D_3 was observed at 84 °C, while the blank beverage exhibited peaks at 89 °C and 250 °C. In contrast, the fortified jamun juice (VDFJJ) showed peaks at 110 °C and 267 °C, indicating a slight increase in thermal stability, likely due to the incorporation of vitamin D_3 carriers within the emulsion system (Bunjes & Unruh, 2007). This peak shift may also be due to the formation of a stable emulsion or due to interactions between vitamin D and the juice components. Similar melting behavior has been previously reported for nano-phytosome formulations of vitamin D_3 (Molaveisi et al., 2020) and was corroborated by our prior study (Jan et al., 2022), which demonstrated comparable melting peaks for vitamin D_3 in a nanoemulsion system.

3.7. Stability studies

3.7.1. Changes in pH, acidity, and total soluble solids of VDFJJ

The pH, titrable acidity, and soluble solids of VDFJJ samples during three months of storage were evaluated at 15-days intervals and are presented in Table 2. The pH and TSS values of the fortified beverage decreased from 4.28 ± 0.23 to 3.21 ± 0.18 and10.7 ± 0.51 to 10.1 ± 0.40 respectively. The titrable acidity of VDFJJ increased from 0.33 ± 0.08 to 0.45 ± 0.09.The increasing trend of acidity may be because of the formation of organic acids by ascorbic acid degradation or due to high phenolic content present in jamun juice. The decrease in the pH can be attributed to the simultaneous increase in acidity.

3.7.2. Anthocyanin content of VDFJJ

Anthocyanins are important phenolic compounds reported to have antioxidant, anti-inflammatory anti-cancer, and anti-microbial properties, and their role in the prevention of various diseases such as diabetes, has been well established (Enaru et al., 2021). The total anthocyanin content of VDFJJ was found to be 1.73 ± 0.12 mg/mL. Over a three-month storage period, this content decreased to 0.95 ± 0.06 mg/mL (Table 2). Similar findings have been observed in previous studies. Ghosh et al. (2017) reported an anthocyanin content of approximately 195 mg/100 g in the jamun pulp, while Shahnawaz et al. (2009) reported an anthocyanin content of 185.35 mg/100 g in jamun juice. Furthermore, Jebitta et al. (2016) noted a decrease in anthocyanin levels from 7.25 mg/g to 6.53 mg/g in freeze-dried jamun samples during storage. These studies highlight the gradual degradation in anthocyanin content in jamun-based products over time, likely due to storage related factors such as oxidation, temperature, and light exposure. Anthocyanins are also sensitive to environmental conditions, that can lead to degradation and reduced pigment stability over time.

Table 2
Changes in physiochemical and microbiological parameters of vitamin D fortified jamun juice (VDFJJ) during storage.

No. of storage days	pH value	Acidity (% citric acid)	Total soluble solids (^{0} Brix)	Anthocyanin (mg cyanidin /mL)	Total plate count (CFU/ mL)	Total yeast and mold count (CFU/ mL)
0	4.28 ± 0.23	0.33 ± 0.08	10.7 ± 0.51	1.73 ± 0.12	14×10^2	5×10^2
15	4.14 ± 0.15	0.34 ± 0.01	10.5 ± 0.43	1.56 ± 0.05	14×10^2	5×10^2
30	3.85 ± 0.09	0.35 ± 0.02	10.3 ± 0.68	1.39 ± 0.25	18×10^2	7×10^2
45	3.62 ± 0.11	0.37 ± 0.04	10.3 ± 0.44	1.26 ± 0.16	21×10^2	10×10^2
60	3.47 ± 0.17	0.39 ± 0.06	10.2 ± 0.52	1.17 ± 0.13	3×10^3	16×10^2
75	3.32 ± 0.33	0.42 ± 0.05	10.2 ± 0.45	1.06 ± 0.10	9×10^3	26×10^2
90	3.21 ± 0.18	0.45 ± 0.09	10.1 ± 0.40	0.95 ± 0.06	17×10^3	34×10^2

3.7.3. Vitamin D_3 during storage of VDFJJ

As shown in Fig.4, vitamin D quantity declined during the storage period of three months from 3992 ± 12.7 IU (4000 IU) to 2440 ± 40.2 IU. The stability of vitamin D can be affected by various factors like pH, heat, oxidation, and the tendency to phase separation (McClements et al., 2009). Further flocculation, coalescence, sedimentation, swelling, and rupture of droplets add to emulsion instability (Ozturk, 2017). Moreover, the stability of vitamin D can be affected by the components of the food matrix. Fruit juices are usually acidic, and acid presence isomerizes vitamin D_3 to isotachysterol which is very sensitive to oxygen and thus causes degradation (Mahmoodani et al., 2017). In previous studies, vitamin D_3 was reported to be stable in various fortified foods: milk for 21 days (Hanson & Metzger, 2010), yogurt for 21 days (Jafari et al., 2016), irradiated fresh mushrooms for 4 days (Malik, Jan, Al-Keridis, et al., 2022), soybean oil for 50 days (Hemery et al., 2015), white chocolate for 120 days (Didar, 2021) and 173 days in wheat flour (Bajaj & Singhal, 2021). After three-month study the content of vitamin D in VDFJJ as indicated by the HPLC chromatograms was higher than the recommended daily allowance (RDA) of 600–800 IU which indicates the suitability of jamun juice for vitamin D fortification.

3.7.4. Colour analysis

Colour is one of the most important factors in the selection of fruit juices for commercial use. The jamun juice extracted was tested for colour response in terms of *L, a, b, c, h,* and *dE* values over three-month storage at a 15-day interval. On day 0 “L” value of VDFJJ was found to be 23.2, the “a” value was 17.9, the “b” value was 4.9, the “c” value was

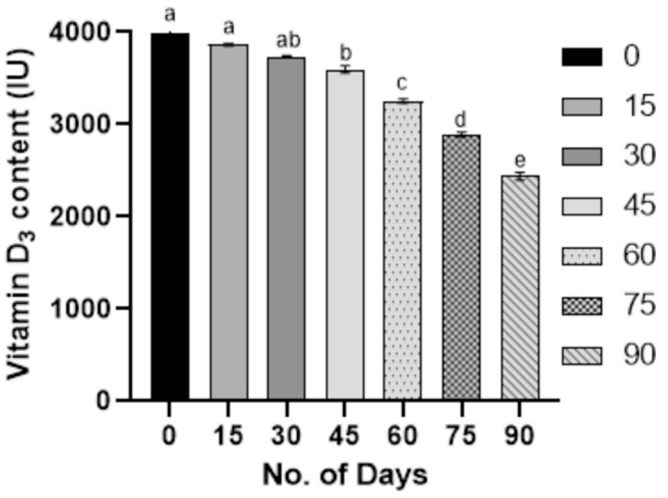

Fig. 4. Vitamin D_3 concentration of the VDFJJ during three months of storage, error bars represent standard deviation for $n = 3$. The experimental values that do not have a common superscript are significantly different ($p < 0.05$).

18.6, and the "h" value was 15.3. In the storage period, an increase in the *L* value was observed while the *a* and *b* values decreased. At the end of three months, the change in VDFJJ, *L, a, b, c, h,* and *dE* values were 32.1, 13, 15.9, 21, 49.9, and 14.7 respectively. The change in values is given in Table 3 and it can be observed that there was not any unfavorable change in the colour of the fortified juice during the storage. The reason for the increase in the "L" value (lightness) may be due to the settling down of some suspended colored particles which are also indicated by the decrease in total soluble solids of the juice. The decrease in colour values (a & b) may be due to the degradation of the anthocyanins. A comparative storage study on jamun juice was carried out by Ghosh et al. (2019) in which they have also reported an increase in the lightness (*L* value) and decreased a & b value during the two-month storage. In another study conducted by Bhatt et al. (2020) in the preparation of wild jamun syrup, the same trend of *L, a,* and *b* values were reported.

3.7.5. *Microbial stability of VDFJJ during storage*

The total bacterial count was enumerated to check the deterioration by various kinds of bacteria present in the jamun juice. A microbial study of jamun juice was done every 15-day intervals for 90 days (Table 2).Initially, the total plate count of VDFJJ was 14×10^2 CFU/mL and the yeast and mold count was 5×10^2 CFU/mL. At the end of 90 days, the TPC and TYC of VDFJJ were found to be 17×10^3 and 34×10^2 CFU/mL, respectively. The log CFU/mL value of the juice sample in the order of 10^6 or more is considered spoiled. A similar data trend of microbial analysis were found by Sridhar et al. (2017) in the formulation of jamun juice blended with guava fruit and Ghosh et al. (2019) for storage study of jamun juice.

4. α-amylase and α-glucosidase activity of the VDFJJ

The amylase inhibitory activity of jamun juice extract demonstrated an IC_{50} value of 110 ± 0.08 μg/mL, indicating its strong antidiabetic potential. This low IC_{50} value reflects the extract's effective inhibition of α-amylase, which is crucial in reducing the breakdown of starch into glucose, thereby mitigating postprandial hyperglycemia. The IC_{50} value observed in this study is considerably lower than that reported by Gajera et al. (2017), who found an IC_{50} value of 270 μg/mL, suggesting that the jamun juice extract used in our study may possess a higher concentration of bioactive compounds responsible for inhibiting α-amylase activity.

Similarly, the IC_{50} value for α-glucosidase inhibition was 134 ± 1.12 μg/mL, further supporting the antidiabetic efficacy of jamun juice. This finding is consistent with the study by Kaur & Bansal (2020), who reported an IC_{50} value of 148 ± 0.01 μg/mL for α-glucosidase inhibition in *Syzygium cumini* extract. The slight variation in IC_{50} values may be attributed to differences in extraction methods, sample preparation, or the phytochemical composition of the jamun fruit used in various studies.

The strong inhibition of both α-amylase and α-glucosidase enzymes by jamun juice extract underscores its potential as a natural antidiabetic agent. These enzymes are key targets in the management of type 2 diabetes, as their inhibition reduces the absorption of carbohydrates and helps control blood sugar levels. The important constituents present in the black jamun are anthocyanins, polyphenolic compounds (glucoside, anthocyanin, ellagic acid, isoquercetin, jambolin and jambosine) have been reported to reduce blood glucose, however among these, jambolin and jambosine, appreciably alters the hydrolysis of carbohydrates into the simpler sugars and thereby improving the diabetic condition (Ayyanar & Subash-Babu, 2012). The results of this study, in conjunction with previous research, highlight the promising role of jamun juice in developing functional foods or nutraceuticals aimed at managing diabetes.

Table 3
Changes in colour characteristics of VDFJJ during storage.

Colour Parameters	Day 0	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90
L	23.2 ± 0.2^a	24.1 ± 0.4^a	25.6 ± 0.3^a	27.2 ± 0.5^b	28.6 ± 0.4^b	31 ± 0.7^c	32.1 ± 0.3^c
A	17.9 ± 0.3^a	17.9 ± 0.2^a	17.4 ± 0.3^a	17.1 ± 0.3^a	13.8 ± 0.4^b	13.1 ± 0.3^c	13 ± 0.3^c
B	4.9 ± 0.1^a	8.1 ± 0.1^b	8.4 ± 0.4^b	9 ± 0.3^b	15.2 ± 0.2^c	15.2 ± 0.2^c	15.9 ± 0.3^c
C	18.6 ± 0.4^a	19.3 ± 0.3^a	19.6 ± 0.3^a	19.8 ± 0.4^a	20 ± 0.3^a	20 ± 0.4^a	21 ± 0.4^b
H	15.3 ± 0.2^a	24.7 ± 0.6^b	25.4 ± 0.4^b	27.8 ± 0.4^b	45.6 ± 0.4^c	48.4 ± 0.4^c	49.9 ± 0.2^d
dE	0.0 ± 0.0	3.8 ± 0.1^a	4.2 ± 0.2^a	5.8 ± 0.1^b	12.6 ± 0.4^c	13.8 ± 0.3^c	14.7 ± 0.6^d

L signifies lightness, a-red to green colour, b-yellow to blue colour, C denotes colour saturation, h indicates the relative amounts of different colours and ΔE entire difference with reverence to control group (day 0). Data is expressed as mean values of triplicates ± SD. Values within the same row with different superscript letters are significantly different at $p < 0.05$ based on Tukey's post hoc test.

4.1. *Release kinetics of vitamin D_3 in simulated gastric and intestinal fluids*

One of the objectives of nanoemulsion formulation for the fortification of jamun juice is to prevent the degradation of the essential micronutrient/fat-soluble vitamins (vitamin D_3) during its passage through the gastrointestinal tract and to enhance its absorption in small intestines, thereby increasing its bioavailability (Ozturk et al., 2015). Therefore, the strength of vitamin D_3 in the VDFJJ was evaluated in simulated gastrointestinal fluids.

In shown in Fig. 5, the percentage release of vitamin D_3 in the simulated gastric fluid (SGF) was approximately 26.30 % after 2 h, while in simulated intestinal fluid (SIF) the release reached 78.15 % after 6 h. The release behavior is suggested to be largely influenced by the interface between the transporter matrix and the release medium (Jan et al., 2022). Additionally, the release rate can be influenced by the interaction of vitamin D_3 with other ingredients in the beverage. Previous research has reported the release percentage of vitamin D_3 in GSF and ISF as 7 % and 93 % (Molaveisi et al., 2020). Another study by Park et al. (2017) reported values of 4 % and 80 % in SGF and SIF respectively.

The release of vitamin D_3 from VDFJJ in simulated gastro-intestinal fluid was also analyzed kinetically using several models, including Zero-order, First-order, Korsmeyer-Peppas, Higuchi, Pappas-Sahlin, Hopfenberg, and Quadratic models. The corresponding model parameters and correlation coefficients are presented in Table 4. Among these models, the Korsmeyer-Peppas model was found to be the best fit, with an **R^2 value of 0.97**. The release mechanism of vitamin D_3 from VDFJJ is

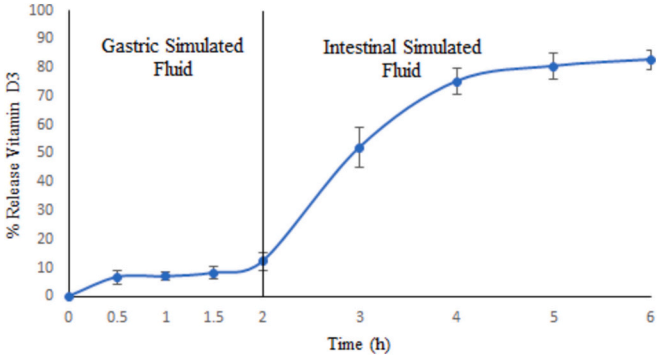

Fig. 5. Vitamin D_3 release % from the VDFJJ in the simulated gastro and intestinal fluids.

Table 4
Model parameters of vitamin D_3 release from VDFJJ.

Models	Parameters	Values
Zero order	k R^2	15.714 0.9278
First order	k R^2	0.345 0.9235
Higuchi	k R^2	28.605 0.8918
Korsmeyer-Peppas	k n R^2	21.423 0.768 0.9742
Pappas Sahlin	k_1 k_2 R^2	12.091 8.882 0.9386
Hopfenberg	k n R^2	0.121 1.914 0.9231
Quadratic	k_1 k_2 R^2	−0.008 0.179 0.9213

explained by the exponent parameter nnn in the Korsmeyer-Peppas model. When $n < 0.45$, the release is governed by Fickian diffusion; when $n \geq 0.89$, dissolution is the dominant mechanism and when $0.45 < n \leq 0.89$ both Fickian diffusion and dissolution mechanisms occur simultaneously (Fathi et al., 2013). In the present study, as shown in Table 4, the release of vitamin D_3 from VDFJJ in simulated gastrointestinal fluids is driven by a combination of Fickian diffusion and dissociation mechanisms.

4.2. Bioavailability of vitamin D from VDFJJ in animal models

The quantification of the serum 25(OH)D_3 was done by using the Chemiluminescent Immunoassay (CLIA) method. The vitamin D deficient group showed relatively low levels of the serum 25(OH)D_3, calcium, phosphorous and alkaline phosphatase as compared to the vitamin D deficient animal group fed with the VDFJJ. It was observed that the encapsulated vitamin D in the jamun juice raised the serum levels of vitamin D in the animal model after dosage with VDFJJ for one month. From Table 5, it can be seen that serum 25(OH)D_3 levels raised in group 3 from 16.15 ± 1.98 at 0th week to 56.96 ± 5.42 at 4th week of dosage. While as in group 2, a relatively low increase serum 25(OH)D_3 levels was observed from 13.72 ± 1.63 at 0th to 16.50 ± 2.46 at 4th week. In group 4, there was increase in the serum 25(OH)D_3 levels from 12.32 ± 1.58 at 0th week to 62.22 ± 5.96 at 4th week. The increase in the serum levels of vitamin D in group 3 and group 4 was high as compared to the control animal group and non-fortified plain beverage. The results also confirm that the vitamin D fortified beverage raised the serum 25(OH)D_3 levels in the animals. In Table 6, the results display the effect of the VDFJJ on the serum values of PTH (pg/mL), ALP (U/L), calcium (mg/dL) and phosphorous (mg/dL) levels in animal models. It was seen that PTH levels in group 3 and group 4 showed a decrease in the PTH levels from 74.35 ± 4.58 at 0th week to 28.80 ± 2.08 at 4th week and from 67.86 ± 6.63at 0th week to 22.22 ± 2.43 at 4th week pg/mL respectively as compared to the control and the plain beverage treated group where an increase in the PTH levels was seen. The values of ALP was also seen decreasing in group 3 and group 4, a decrease in the serum ALP values from 142.62 ± 21.53 at 0th week to 69 ± 13.15 at 4th week and from 155.56 ± 15.46 at 0th week to 61 ± 15.39 at 4th-week U/L respectively which was significantly lower than the control group and plain beverage treated group. The serum calcium (mg/dL) values increased in group 2 (5.08 ± 1.88 at 0^{th}day to 9.96 ± 1.40 at the 28th day), group 3 (5.92 ± 1.18 at 0^{th}day to 10.74 ± 1.55 at 28th day) and group 4 (6.79 ± 1.45 at 0^{th}day to 10.53 ± 2.78 at 28th day) as compared to the control group. The phosphorous levels (mg/dL) were increasing in the group 2 (1.26 ± 0.20 at 0^{th}day to 3.41 ± 0.79 at 28th day), group 3 (1.22 ± 0.32 at 0^{th}day to 3.24 ± 0.92 at 28th day) and group 4 (2.16 ± 0.24 at 0th day to 2.87 ± 0.98 at 28th day) as compared to the control group (1.32 ± 0.14 0th day to 1.58 ± 0.1 at 28th day). The results indicate that the VDFJJ had a significant effect on the biochemical parameters along with the serum levels of 25(OH)D_3.

Table 5
Serum 25-hydroxyvitamin D levels (ng/mL) before and after 4-weeks of feeding period.

Groups	0th Week	1st Week	2nd Week	3rd Week	4th Week
G-1	15.42 ± 2.23[a]	14.18 ± 1.08 [a]	12.86 ± 2.36 [a]	15.21 ± 1.93 [a]	17.67 ± 3.12 [a]
G-2	13.72 ± 1.63 [a]	18.32 ± 2.93 [a]	13.66 ± 2.77 [a]	17.71 ± 3.31 [a]	16.50 ± 2.46 [a]
G-3	16.15 ± 1.98 [a]	23.18 ± 2.55 [b]	35.12 ± 4.64 [c]	48.72 ± 6.12 [d]	56.96 ± 5.42 [d]
G-4	12.32 ± 1.58 [a]	22.05 ± 4.67 [b]	38.42 ± 3.84 [c]	53.43 ± 3.32 [d]	62.22 ± 5.96[e]

Data were expressed as mean ± SD where n = 6. One-way ANOVA with Tukey's post hoc test was used to test the significant difference. The experimental values that do not have a common superscript are significantly different ($p < 0.05$).

The primary role of vitamin D_3 is mainly associated with bone health but its deficiency has found its role in various non-communicable diseases and other metabolic syndromes (Bikle, 2014). In our study, after supplementation with vitamin D3-fortified jamun juice, there was a marked improvement in serum 25(OH)D_3 levels in vitamin D-deficient animal models, indicating enhanced vitamin D absorption. This increase positively influenced calcium and phosphorus levels, which are essential for bone mineralization and overall skeletal health. Additionally, the reduction in alkaline phosphatase levels suggests an improved bone health and overall metabolism. These findings highlight the efficacy of fortified jamun juice in addressing vitamin D deficiency and promoting overall health. Our finding followed a similar trend as reported by a previous study by Malik, Jan, Haq, et al. (2022) where they had administered oral doses of vitamin D_3 (market sample) and UV-treated mushrooms (vitamin D-rich mushrooms) in vitamin D-deficient animal models.

4.3. Sensory evaluation

The sensory analysis of VDFJJ was focused on evaluating its organoleptic characteristics and overall acceptability to ensure that the fortification process does not compromise the sensory attributes that consumers expect. Jamun juice is cherished for its unique sweet and tangy flavor and vibrant deep purple colour. When fortified with vitamin D_3, it was important to assess whether the added nutrients has any potential effects on its colour stability, flavor profile or texture. Although vitamin D_3 is a tasteless and odorless, generally has minimal direct influence on the flavor or aroma, however, the carrier used for fortification or potential pH changes could alter the mouthfeel or aftertaste. Fig. 6 demonstrates that vitamin D fortification of jamun juice enhances the nutritional value without compromising the sensory qualities. Ensuring that sensory attributes remained intact, which is essential for consumer satisfaction. Thus, the overall acceptability of fortified Jamun juice confirmed the suitability of the vitamin D_3 emulsion system in the fruit beverage.

5. Conclusion

In conclusion, the vitamin D_3-enriched nanoemulsion developed using a low-temperature sonication method demonstrated excellent stability, bioavailability, and compatibility for fortifying beverages. The Vitamin D_3 emulsion used to fortified jamun juice exhibited a particle size of approximately 169 nm, a zeta potential of −26 mV, and promising *in-vitro* antidiabetic activity. It achieved 82 % vitamin D_3 release in simulated gastrointestinal fluids and significantly improved serum 25 (OH)D, PTH, calcium, phosphorus, and ALP levels in vitamin D-deficient animal models, validating its efficacy in addressing vitamin D

Table 6
Serum values of biochemical parameters of animals before and after feeding with vitamin D_3 fortified jamun juice (VDFJJ).

Group	PTH (pg/mL)		ALP (U/L)		Calcium (mg/dL)		Phosphorous (mg/dL)	
	0th Day	28th Day	0th Day	28th Day	0th Day	28th Day	0th Day	28th Day
G-1	64.18 ± 4.53^a	69.24 ± 5.21^a	154.14 ± 28.11^a	157 ± 25.33^a	5.45 ± 2.42^a	6.38 ± 1.18^a	1.32 ± 0.14^a	1.58 ± 0.17^a
G-2	77.95 ± 5.42^a	71.85 ± 7.11^b	170.10 ± 32.12^a	138 ± 14.32^b	5.08 ± 1.88^a	9.96 ± 1.40^b	1.26 ± 0.20^a	3.41 ± 0.79^b
G-3	74.35 ± 4.58^a	28.80 ± 2.08^b	142.62 ± 21.53^a	69 ± 13.15^b	5.92 ± 1.18^a	10.74 ± 1.55^b	1.22 ± 0.32^a	3.24 ± 0.92^b
G-4	67.86 ± 6.63^a	22.22 ± 2.43^b	155.56 ± 15.46^a	61 ± 15.39^b	6.79 ± 1.45^a	10.53 ± 2.78^b	2.16 ± 0.24^a	2.87 ± 0.98^b

Data were expressed as mean ± SD where n = 6. t-test was used to test the significance level. The experimental values that do not have a common superscript are significantly different ($p < 0.05$).

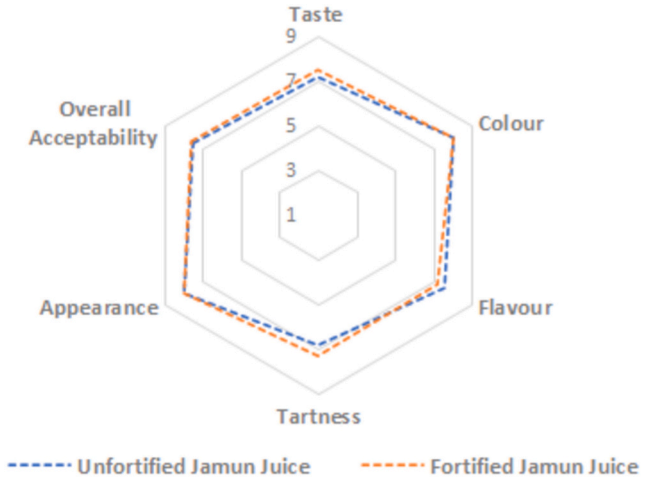

Fig. 6. Sensory attributes of unfortified jamun juice and vitamin D_3 fortified jamun juice.

deficiency. Sensory analysis affirmed its consumer acceptability in terms of taste, colour, and overall acceptability. Vitamin D_3 demonstrated stability within the juice matrix, with only a moderate reduction over three months. The fortified beverage will offer a cost-effective and functional nutritional solution, particularly for diabetic populations and those seeking to enhance immunity and bone health. The combination of mineral and phenol-rich profile of jamun with vitamin D_3, offers an innovative approach in combating micronutrient deficiencies. While Wistar rats are a valuable model for vitamin D and diabetic research, translating these findings to human studies remains essential to confirm clinical relevance. Future research could explore the fortification of other fruit juices, assess the long-term stability of such formulations, and evaluate their impact in human populations to further expand the potential of functional beverages in combating micronutrient deficiencies and associated health challenges.

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Author contribution

Yasmeena Jan, Reem Binsuwaidan, Muneeb Malik and Bibhu Prasad Panda contributed to study concept and design, collected/analyzed data, project administration and drafted the original manuscript;, Mifftha Yaseen, Mohd Adnan, Sayeed Ahmad, Nawaf Alshammari, Mohd Adnan and Syed Amir Ashraf, contributed to methodology, data curation, investigation, visualization, analysis, review and editing; Reem Binsuwaidan, Mohd Adnan, Muneeb Malik and Bibhu Prasad Panda and contributed to critical revision of the manuscript, validation, formal analysis and study supervision. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Yasmeena Jan: Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Reem Binsuwaidan:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Muneeb Malik:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Formal analysis. **Mifftha Yaseen:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Sayeed Ahmad:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation. **Nawaf Alshammari:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Mohd Adnan:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Data curation. **Syed Amir Ashraf:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Bibhu Prasad Panda:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

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.

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Data availability

The information supporting this study is available in this article.

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