Preparation and Characterization of Liposomal β-Caryophyllene (Rephyll) by Nanofiber Weaving Technology and Its Effects on Delayed Onset Muscle Soreness (DOMS) in Humans: A Randomized, Double-Blinded, Crossover-Designed, and Placebo-Controlled Study

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ABSTRACT: Delayed onset muscle soreness (DOMS) is a complex spreading out, which is related to swelling of muscles, tenderness, rigidity, pain, disruption of muscle fiber, alteration in the kinematics of joint, acute tissue damage, and reduction in power and strength. β-Caryophyllene (BCP), a potent phytocannabinoid, could play an important role in managing DOMS because of its wide diversity of biological activities, particularly its anti-inflammatory activity; however, its poor stability in light, temperature, high volatility, and insolubility can restrict the medical practices. In this study, liposomal β-caryophyllene (Rephyll) was designed and established in powder form constructed by the nanofiber weaving technology to improve the bioavailability of BCP with improved stability. Rephyll was characterized by Fourier transform infrared spectroscopy, transmission electron microscopy, and differential scanning calorimetry studies. Encapsulation efficiency, loading capacity, and in vitro release studies revealed that Rephyll can be an auspicious drug delivery arrangement for BCP. The effects of Rephyll were evaluated by a randomized, double-blinded, crossover-designed, placebo-controlled study. The oral consumption of Rephyll significantly reduced the pain visual assessment score, revealing that Rephyll effectively reduced DOMS with improved recovery without any side effects due to the bioavailable form of the phytocannabinoid BCP in the liposomal powder formulation.

INTRODUCTION

Delayed onset muscle soreness (DOMS) and damage in muscles are the multifactorial route and probable systematic concepts that take into account together the physiological/functional and biochemical components, which are associated with disruption of muscle fiber, muscle pain, alteration in the kinematics of joints, weakened range of motion, acute damage in tissues, and decreased strength.1 After an intense session of exercise, muscle soreness decreased the power and performance. Therefore, recovery from DOMS and muscle damage is becoming increasingly important so that any sports person or athlete or any individual with interest in exercise may undergo training more frequently to increase long-term performance.2 Even though the possible mechanisms responsible for these warning signs are not totally tacit, the damage in structures affected by eccentric exercise is considered to be due to disassociation of myofilaments, broadening and issuing of z-line, break of the concentration–excitation linking route, and an immune reaction that produces a gathering of mononuclear cells. Various treatment methods are already in use to diminish destruction or develop recovery from DOMS, for instance,
curative modalities such as massage, stretching, and cryother-
apy and pharmacological medications such as nonsteroidal
anti-inflammatory drugs and dietary supplements.5,4

In recent years, phytochemicals have accredited consid-
eration for their various biological activities. Among the
phytochemicals, phytocannabinoids have expanded massive
consideration for their anti-inflammatory, cardioprotective,
and anticancer properties.5–8 Among the phytocannabinoids, β-
caryophyllene \([C_{15}H_{24}]\) (BCP) \([(1R,4E,9S)-4,11,11\text{-trimethyl-}
8-methylidenebicyclo[7.2.0]undec-4-ene]\) (Figure 1), a bicyclic

![Figure 1. Chemical structure of β-caryophyllene.](image)

sesquiterpene plentifully offered in spices, particularly \(Piper
nigrum\ L., Cinnamomum verum, Origanum vulgare, and Eugenia
caryophyllata\) has attracted substantial attention because of its
nutritional readiness and extensive care. The FDA has
approved BCP as an adjuvant or flavoring agent as well as a
pharmaceutical representative because of its various pharma-
ceutical activities, moreover, which has a woody spicy odor and
slightly yellowish color; however, it has low water solubility.9,10

In recent years, BCP has gained interest with extensive care
due to its broad biological activities comprising antimicrobial,
anticancerogenic, anti-inflammatory, antioxidiant, antispasmod-
ic, gastric cytoprotection, and anesthetic effects.5–10 BCP has
been reported as a potential phytocannabinoid due to its anti-
inflammatory and it diminishes arthritis that was marked by an
index of arthritis, a volume of paw besides the preservation of
biochemical factors. BCP has noticeable anti-arithmetic action in
adjuvant arthritic rats due to its anti-inflammatory activity via
inhibition of joint inflammation and destruction and NF-xB activity.11 Oral administration of BCP reduced inflammatory
pain responses, weakened mechanical allodynia and thermal
hyperalgesia, and attenuated spinal neuro-inflammation.12 BCP
inhibited tissue damage and inflammation in model of colitis
and nephrotoxicity.13,14 Co-administration of BCP with
methotrexate and leflunomide was used as an effective therapy
for rheumatoid arthritis.15 The healing activity of BCP was
highly effective because of its anti-inflammatory property. The
anti-inflammatory properties of BCP could be favorable to
treat and manage the inflammation-related diseases.

However, BCP is highly volatile, insoluble in water, and
sensitive to oxygen, humidity, light, and high temperature.16,17
Among them, the insoluble nature in water may considerably
attenuate the bioavailability of the drug and therefore inhibit
its additional application in nutraceutical, pharmaceutical, and
functional food fields. Therefore, creating a drug delivery
system, particularly for BCP, to enhance its solubility and
dissolution rate, likewise to increase the bioavailability, has
been an essential task in pharmaceutical and nutraceutical
development.17 Recently, nanocarriers denote an inventive
strategy to optimize the performance of formulations fabricated by the BCP and overcome these core limita-
tions.18,19

Liposomes are sphere-shaped vesicles made up of an
aqueous center and a phospholipid bilayer membrane and
have been extensively scrutinized as a delivery scheme for both
hydrophobic and hydrophilic molecules. The main advantage
of utilizing phospholipid preparations as a drug delivery system
for oral consumption is that the bioactive molecules that are
susceptible to collapse in the gastrointestinal tract by enzymes
are probably protected through a liposomal layer, although one
of the main limitations of utilizing liposomes in place of drug
carriers is their rapid dismissal after circulation of blood and
reticuloendothelial arrangement of macrophages. Moreover, the
physicochemical uncertainty of liposomes indicates complications, particularly the degradation, aggregation,
ocidation, and hydrolysis of phospholipids. Furthermore, the
available liposomal formulations are in the liquid/suspension
system and stabilize through a water-in-oil suspension in place of
vehicle-like partitions designed for encapsulations, even
though they have slight stability because of the unsystematic
behavior of the collapsible phospholipid bilayer in the presence
of water, usually leading to randomness in both size and shape.
A powder form of liposomal formulation could be a good
remedy to resolve these difficulties even after drying.9,20–22

The study aimed to formulate a powder form of liposomal β-
caryophyllene formulation (Rephyll) by nanofiber weaving (NFW)
technology through well-organized nanofiber (NF) fabrication using homogenization with high pressure followed
by a spray drying process that can expand the utilization
outline of BCP, particularly in the pharmaceutical and
nutraceutical industries. The characterizations of Rephyll
were carried out by Fourier transform infrared spectroscopy
(FT-IR), transmission electron microscopy (TEM), and
differential scanning calorimetry (DSC). The other important
objectives of this study are to assess the effects of Rephyll on
DOMS compared to placebo on pain, isometric strength, and
joint flexibility and to evaluate the effects on blood parameters
and the incidence of adverse events.

### MATERIALS AND METHODS

**Materials.** β-Caryophyllene, derived from black pepper,
and turmeric nanofiber were supplied by Plant Lipids Private
Limited, Cochin, Kerala, India. Phospholipids were acquired
from Shankar Soy Concepts, Indore, India. Organic solvents
were of LC–MS, GC–MS, and HPLC grade and standards
and other chemicals were procured from Merck India.
Millipore Milli-Q distilled water was utilized throughout the
study.

**Preparation of Rephyll.** In this study, a powder form of
phospholipid vesicles was prepared via well-ordered NF
fabrication by nanofiber weaving (NFW) technology under
homogenization with high pressure and spray drying process
to remove water. The NFW technology can be applied to protect
the functional properties with increased stability of the
bioactive molecules, sustain release of the active molecules at
a specific period intended for a preferred target, and enhance
the bioavailability of bioactive molecules with improved
healthiness. The NFW technology is one of the methods
that has the supreme prospective to enhance the dissolution of
low-soluble bioactive molecules. Moreover, the NFW technol-
yogy is a simple, low-budget process and is profitably feasible for
industrial production.

Rephyll was formulated by blending of β-caryophyllene,
phospholipids, turmeric nanofiber (TNF), and bisacrona as
an additional anti-inflammatory agent23 and modified food
starch by a distinctive invention of NFW technology in this way initially; phospholipids (20 g) were dissolved in Millipore water (1 L) at 80 °C. β-Caryophyllene (10 g) with bisacurone (1 g) was added to that solution for encapsulation onto the liposomal bilayer by continuous stirring. The subsequent mixture was agitated for 30 min; after that, 20 g of TNF was added with 49 g of modified food starch as a stabilizing agent to maintain the anchoring effect inside the liposomal structure. This preparation was homogenized with a pressure range of 50–500 bars utilizing a PRIMIX-Homomixer Mark II.L5 homogenizer and the process was continued three times. The prepared formulation was dehydrated through the spray drying method (Ohkawara Kakohki Co., Ltd., Japan, model L-8) with an inlet temperature in the range between 175 and 185 °C and outlet temperature in the range between 75 and 85 °C. The liposomal β-caryophyllene (Rephyll) thus achieved was preserved in an amber bottle and kept at room temperature. This liposomal β-caryophyllene formulation was designed by the NFW technology based on the fabrication of phospholipids and β-caryophyllene with bisacurone by NF through the electrostatic interactions, which has been shown schematically with the proposed mechanism of action in Figure 2. The proposed mechanism of liposomal powder formulation is in this way: since the NF acts as dispersion in the aqueous environment for the period of homogenization, the bioactive molecules are well encapsulated onto the liposomal bilayer and water forms the core of the liposomes. At the time of spray drying, the large vesicle liposomes contract to a smaller size and form small vesicle liposomes. By the reason of the electrostatic interaction between the phospholipids and the NF, the well-structured NF exerts an "anchoring effect", rendering the spherical liposomal arrangement undamaged. With simultaneous addition of water, the small vesicle bulges, and it converts into a large vesicle as earlier.

**Analysis of β-Caryophyllene.** The BCP content in Rephyll was measured by a gas chromatograph (Agilent 7890A), combined with a mass spectrometer 5975CMS, connected with an Agilent 19091S-433 DB MS (325 °C; 30 m × 250 μm × 0.25 μm). The column inlet temperature was kept at 250 °C. The column temperature was held at 100 °C for 1 min, then raised to the first temperature ramp of 180 °C at a level of 20 °C/min, and maintained for 1 min. Then, it was computerized to the second column temperature ramp of 250 °C at a level of 25 °C/min and kept in that closing temperature for 1.2 min. Helium gas was applied as the carrier gas at a flow rate of 1 mL/min. The ions using masses of 93, 133, 79, and 91 were monitored for the BCP molecule. The method was standardized and a calibration curve was plotted using standard BCP. Rephyll was weighed (~50 mg), diluted with 50 mL of methanol, filtered, and injected. The purity was calculated by using the calibration curve. All samples were analyzed in triplicate.

**Physical Characterization of Rephyll.**

**Moisture Content and Hygroscopicity.** The moisture content was assessed by the AOAC method. A triplicate sample of Rephyll (20 g) was weighed and then dehydrated in a vacuum oven at 70 °C. The drying and weighing processes were continued until constant weight was reached. The hygroscopicity study of Rephyll was evaluated by equal dispersion of 1 g of the samples on Petri plates to allow for a high surface area between humid air and the samples. Every sample in the plates was located in desiccators under the settings of 76% relative humidity utilizing nitric acid at 23 °C. The weight expansion of the samples was noticeably lesser for the next 90 min. Although hygroscopicity is established on the steadiness of the content of moisture, to relate hygroscopicities, the weight increases per gram of powder samples after being exposed to the atmosphere with comparative moisture of 76% for 90 min were assessed.

**Degree of Caking (DC).** The samples were dried in a drying oven at 70 °C; after cooling down, the dry samples were weighed and relocated into a sieve with a size of 300 mm. The sieve was then agitated for 5 min in an agitating apparatus. The weight of the residual powder in the sieve was measured. The degree of caking was calculated as

\[ DC = \frac{b}{a} \times 100 \]  

where DC is the degree of caking (%), \( a \) is the quantity of powder used in sieving, and \( b \) is the quantity of powder that stayed on the sieve after sieving.

**Bulk Density.** Bulk density (g/mL) was assessed by the addition of 10 g of sample into a blank 100 mL graduated cylinder and the cylinder was positioned on a circle stand. The circle stand was adjusted to facilitate the positioning of the cylinder; once the bottom of the cylinder is elevated to touch the circle, the base surface of the cylinder is accurately 1 in. from the bottom of the circle stand. The ratio of the sample

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**Figure 2.** Schematic representation of preparation of liposomal β-caryophyllene (Rephyll) using nanofiber weaving (NFW) technology.
mass and the volume occupied in the cylinder defines the bulk density values.\textsuperscript{28}

**Characterization of Rephyll.** Fourier transform infrared spectroscopy (FT-IR) spectra of BCP and Rephyll were recorded with a JASCO FT/IR-460 Plus instrument in the range between 400 and 4000 cm\(^{-1}\) by 32 scans per sample. The morphology of Rephyll was assessed with transmission electron microscopy (TEM) (JEOL-2100, JEOL, USA). The samples were diluted with a Whatman filter and then archived in the dossiers of study documents. The study was approved and monitored by the ethics committee to safeguard the rights, safety, and well-being of all experimental subjects. The authenticity and credibility of the data were well maintained by means of regular site monitoring and audits. Written informed consent was contracted from every subject earlier than the commencement of the clinical study. The subject was well learned with adequate information concerning planned clinical trial, comprising benefits and risks involved. All queries from subjects and/or relatives were resolved before signing informed consent. Signed and dated informed consents and any other related documents of all subjects have been archived in the dosiers of study documents. The study was registered with Clinical Trials Registry India (clinicaltrials.gov) (CTRI/2019/05/019366).

**Supplementation.** The unique natural pain management formulation incorporates the phytocannabinoid β-caryophyllene (derived from black pepper) using cutting-edge Zeal technology; Rephyll was provided by Aurea Biolabs (P) Ltd., Cochin, Kerala, India. The placebo contained a food grade starch, which was visually similar to Rephyll. The subjects received a single oral 500 mg dosage of one of the products in capsule form.

**Study Design and Plan.** The current study was a double-blinded, randomized, and placebo-controlled trial conducted in accordance with Good Clinical Practice and ICH guidelines. It was designed to assess the effects of Rephyll in comparison with placebo for improvements in healthy male subjects with age in the 19 to 29 year range. A total of 20 eligible participants who met the specified inclusion/exclusion standards were enrolled in the study and randomized in almost 1:1 ratio (\(N = 10\) per group) to receive either Rephyll or placebo, which were crossed over to the second treatment group after a washout period of 2 weeks. They were evaluated over four visits for predetermined efficacy variables. The total study duration was 23 days during which all subjects were closely monitored for protocol compliance as well as assessed for efficacy and safety parameters. Subjects were evaluated at visit 1 (day 0 ± 1), visit 2 (day 1 ± 1), visits 3, 4, and 5 (days 2 ± 1, 3 ± 1, and 4 ± 1), visit 6 (day 18 ± 1), visit 7 (day 19 ± 1), visits 8 and 9 (days 20 ± 1 and 21 ± 1), and visit 10 (day 22 ± 1). All subjects were counseled regularly against the use of prohibited medications. Adverse events were monitored throughout the study period. The scheme of the study design is shown in Figure 3. All subjects were verified for relaxed arm circumference, hanging joint angle, unilateral isometric forearm flexion strength, subjective pain rating, myoglobin concentration, and plasma creatine kinase activity. The testing succeeded instantly after exercise (day 1) and 24 h (day 2), 48 h (day 3), and 72 h (day 4) after a bout of eccentric exercise.

**Inclusion and Exclusion Criteria.** The probable subjects were screened and the eligibility of the subjects was determined on the basis of subsequent inclusion and exclusion criteria.

Determination of Storage Stability. The physical stability of Rephyll was studied over 18 months during the period when it was kept in a sealed glass bottle at 25 ± 3 °C. At specific time intervals, the samples were taken and evaluated for the physical appearance and BCP content before and after storage. All determinations were implemented in triplicate. The stability of BCP was calculated using the formula

\[
\text{stability of BCP (\%)} = \frac{C_t}{C_0} \times 100
\]  

where \(C_0\) is the initial concentration of BCP and \(C_t\) are the concentrations of diffent sampling points in time of BCP.

In Vitro Release Study. In vitro release of Rephyll was investigated using dialysis membrane, as stated by Almeida et al.\textsuperscript{30} by appropriate adaptations. In brief, the samples were diluted five times with the solvent system containing intestinal fluid at pH 6.8 with ethanol in the ratio of 1:9. Then, an adequate amount of the diluted sample was kept in the dialysis bag and placed in an Erlenmeyer flask with 80 mL of the prepared solvent system and maintained at 37 °C under shaking at 100 rpm in the shaker. At predetermined intervals (5, 10, 15, 30, and 60 min and then each hour until 24 h), 1 mL of the aliquot was taken, exchanged by the solvent system, and immediately analyzed by GC–MS. The BCP standard was used as a control. The measurements were performed in triplicates.

The Effects of Rephyll on DOMS in Humans. Ethical Approval. The study procedure was reviewed and accepted by the Institutional Ethics Committee (IEC) from Lifepoint Research Ethics Committee, Pune, Maharashtra, India. This study was designed, conducted, analyzed, and described in agreement with regulatory and ethical guidelines (Declaration of Helsinki, ICH GCP, and Indian GCP, Schedule-Y). It was approved and monitored by the ethics committee to safeguard the rights, safety, and well-being of all experimental subjects. The authenticity and credibility of the data were well maintained by means of regular site monitoring and audits.

Encapsulation Efficiency and Loading Capacity of Liposomal BCP Formulation. Encapsulation efficiency (EE) and loading capacity (LC) were determined in accordance with the method defined by Carneiro et al.\textsuperscript{29} through proper adaptations. Fifteen milliliters of hexane was added to 1.5 g of Rephyll in a glass container with a cap, which was shaken by hand for the extraction of free oil for 2 min at room temperature. The solvent mixture was filtered with a Whatman filter and the powder collected on the filter was cleaned three times with 20 mL of hexane. Then, the solvent was allowed to vaporize at room temperature and then at 60 °C until the persistent weight was obtained. The non-incorporated BCP (surface BCP content) was determined by mass alteration between the initial clean container and that having the extracted BCP remains. The EE (%) and LC (%) were calculated from eqs 2 and 3, respectively.

\[
\text{EE(\%)} = \left(\frac{\text{total BCP content} - \text{surface BCP content}}{\text{total BCP content}}\right) \times 100 \tag{2}
\]

\[
\text{LC(\%)} = \left(\frac{\text{total BCP content} - \text{surface BCP content}}{\text{weight of the sample}}\right) \times 100 \tag{3}
\]
The inclusion criteria included subjects who are male between 19 and 29 years of age with good health, untrained in resistance/power exercise, agreeable, and capable of obeying the protocol and have provided written and dated informed consent to join in the study. The subjects who met any of the subsequent standards were excluded from the study. These include a subject who is contributing in any additional clinical study or has received a trial product within 30 days prior to enrolment, has a history of alcohol or other drug abuse in the past year, and has a substantial history of otherwise recent occurrence of treated or untreated bleeding complaints, diabetes mellitus, high blood pressure, thyroid sickness, tachyarrhythmia, heart illness, kidney infection, or liver sickness. A subject who has an identified allergy or sensitivity to any ingredient in the investigation product, has a history of difficulty swallowing large tablets or pills, has used creatine within 9 weeks prior to screening, has a history of orthopedic surgery or injury within the last year, has any physical condition that reflected a contraindication to the type of exercise performed in the study, and has an abnormal resting ECG was also excluded. A subject who is willing to withdraw from the study at any point in time was also excluded from the study.

Randomization and Blinding. Eligible subjects were randomized in the ratio 1:1 to receive either Rephyll or placebo established on the subsequent accession number according to the randomization plan. The randomization chart was generated by a statistician. The packaging and labeling of investigational products as per randomization chart were done by an independent team not involved in the study conduct. The clinical operation team involved in the study as well as an investigator was blinded for entire randomization as well as the labeling process.

As this was a double-blinded study, subjects as well as a sponsor representatives, particularly the investigator, site study team, and clinical research organization study team, were blinded. Rephyll and placebo were made exactly identical to match organoleptic characteristics such as size, shape, color, and texture to preserve the blinding. They were packed in bottles, exactly similar in size, color, and labeling. The subject identities were organized in a sequential order according to the randomization plan. The blinding codes were protected with tamper-evident closed wrappers with restricted access at the site. Each envelope was mentioned with the subject identity and investigational product allocation. The master randomization plan was sealed in a wrapper and preserved in the trial master file.

Efficacy and Safety Variables. Efficacy variables were assessed at visit 1 (day 0 ± 1), visit 2 (day 1 + 1), visits 3, 4, and 5 (days 2 + 1, 3 + 1, and 4 + 1), visit 6 (day 18 + 1), visit 7 (day 19 + 1), visits 8 and 9 (days 20 + 1 and 21 + 1), and visit 10 (day 22 + 1). Safety laboratory parameters were evaluated at baseline and the end of the study, whereas adverse events were monitored and recorded throughout the study period.

Efficacy Variables. Subjective Pain Rating. The subjective pain rating in the forearm flexor muscles was inspected utilizing a pain intensity scale. The visual assessment score (VAS) scale ranged from 0 (no pain at all) to 10 (extremely intense pain). The subjects were offered with this scale and questioned to find a single score for arm pain with the forearm flexor muscles relaxed and the arm hanging at the side of the subject.

Hanging Joint Angle and Relaxed Arm Circumference. The hanging joint angle (°) between the arm and forearm was inspected by a standard goniometer. For every measurement, the axis of rotation of the elbow joint was aligned with the axis of the goniometer. In addition, the relaxed arm circumference (cm) was determined with a Gulick tape. The maximum girth was determined with the arm horizontally abducted and the forearm extended.

Isometric Strength. Unilateral isometric forearm flexion strength was determined at an elbow joint angle of 115° between the arm and forearm with a Jamar Plus Digital Hand Dynamometer to measure the isometric grip force. Following a warm up of five submaximal muscle actions, the subjects executed two 6 s maximal isometric muscle actions of the forearm flexors, separated by 2 min of rest. The highest torque output from the two maximal muscle actions was chosen as the maximum voluntary contraction (MVC) value. For all the muscle movements, the subjects relaxed in a supine position and practiced a neutral handgrip.

Eccentric Exercise Protocol. For the eccentric exercise protocol, all the subjects completed six sets of 10 maximal eccentric isokinetic muscle actions of the forearm flexors at a velocity of 30° s⁻¹. The subjects were stimulated to deliver an utmost strength during every muscle action. One minute of rest was allowed among every set, and 2 min of break was allowed among the last set and the time 2 isometric strength test.

Blood Muscular Degeneration Biomarkers. The efficacy of the study product was assessed in terms of its effect on the muscular degeneration biomarkers such as creatine kinase (CK) and myoglobin. CK is an enzyme found primarily in skeletal and cardiac muscles. It is elevated in diseases like muscular dystrophy, myopathies, polymyositis, muscle trauma, myocardial infarction, cardiac catheterization, electrical cardioversion, hypothyroidism, and stroke. Myoglobin is a cytoplasmic protein of cardiac and skeletal muscles and passes into the circulation following damage to myocytes. Both of these biomarker levels rise following the induction of DOMS. Hence, the effect of Rephyll to control their amount in the blood was evaluated by hematological analysis.
**Safety Variables.** Safety variables were evaluated at baseline and the end of the study visits. Blood pressure, pulse rate, and body temperature monitoring was done at all visits. Subjects were screened for adverse and serious adverse events at all visits.

**Statistical Analysis.** Continuous variables were summarized per treatment group utilizing summary statistics (number of observations, mean, and standard deviation). Definite values were summarized per treatment group utilizing occurrences and proportions. Student’s paired and unpaired t-tests were applied for assessment of statistical significance of the study results for both treatment groups.

**RESULTS AND DISCUSSION**

**Physical Parameters.** Rephyll is a brown flowing powder, which has registered 4.8 ± 0.6% moisture content, 0.53 ± 0.16 g/g hygroscopicity, 1.8 ± 0.4% degree of caking, and 0.41 ± 0.15 g/mL bulk density. These results are a good sign for the BCP-encapsulated liposomal powder formulation, which are in good agreement with the liposomal product with curcumin.20

**Fourier Transform Infrared Spectroscopy (FT-IR) Studies.** The FT-IR spectra of BCP and Rephyll are represented in Figure 4. The IR spectrum of BCP (Figure 4a) exhibited absorption bands of stretching vibrations at 3067 and 1446 cm⁻¹ of −CH. At 1631 cm⁻¹ appears a stretching vibration associated to the C=O bond. The paired band at 1381 and 1366 cm⁻¹ of symmetrical bending of −CH₃ can be attributed to two methyl groups associated to the similar carbon atom. Methylenic hydrogens make two −CH stretching bands detected at 2924 cm⁻¹ as an asymmetric stretching and 2856 cm⁻¹ as a symmetric stretching that are distinctive of −CH₂ groups. The strong band was detected at 885 cm⁻¹ in the BCP spectrum, which is assigned to out-of-plane deformation vibration of −CH₂ distinctive of the BCP molecule.31 However, this band is not observed in the spectrum of Rephyll (Figure 4b), maybe due to the constraint of the vibration because of the encapsulation process. At 858 cm⁻¹, a band was identified in the spectrum of Rephyll (Figure 4b), which characterized the distinctive composition of cellulose, which is presented in the NF. It is accredited to the twist of glycoside C−H with the ring vibration of −OH bending and authenticates the occurrence of β-glicosidic linkages among the anhydroglucose units of cellulose.29,32 The bands were found in the spectrum of Rephyll formulation, where the encapsulation of BCP onto the lipid bilayer was evidenced by the decrease in the intensity of the bands at 2925 and 2855 cm⁻¹, which are recognized to the symmetric and asymmetric vibrational modes of CH₂ groups; a band at 1154 cm⁻¹ is related to the influences of phosphate groups in the lipids.20,33,34 A broad band detected at 3409 cm⁻¹ is characterized to intramolecular hydrogen bonding in the cellulose of NF and also corresponds to the phenolic O−H stretching vibration in the phospholipids.20 Those changes can be ascribed to the encapsulation of BCP within the liposomal environment and stabilized with NF by the NFW technology.

**Morphology Study of Rephyll.** The surface morphology and shape of Rephyll were scanned by TEM, which is shown in Figure 5. Rephyll has a smooth surface, is spherical-shaped, and is nanosized without any accumulation, indicating the stability of the formulation. The particle sizes of Rephyll are revealed in the range of 450 to 700 nm. The TEM images revealed a distinct gray/dark interface inside and well-rounded transparent phospholipid layer, whereas the inner portion is seen to be darker, which is attributed to the anchoring effect of NF by the NFW technology. Moreover, the TEM images showing the small particles (yellow arrows) onto the liposomal bilayer indicated the good encapsulation of BCP with bisacurone in the liposomal bilayer.

**DSC Analysis.** The DSC thermograms of BCP and Rephyll are shown in Figure 6. The thermogram of BCP (Figure 6a) showed one sharp endothermic event between 90 and 156 °C with the peak temperature at 150 °C assigned to its volatilization.31,38 However, this endothermic peak disappeared after encapsulation of BCP into the liposomes with the anchoring of NF due to the incorporation of BCP on the bilayer of the phospholipids (Figure 6b). Furthermore, the thermogram of Rephyll exhibited three endothermic events.

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**Figure 4.** FT-IR spectra of (a) BCP and (b) Rephyll.

**Figure 5.** Transmission electron microscopy images of Rephyll.

**Figure 6.** DSC thermogram of (a) BCP and (b) Rephyll.
The first occurred between 60 and 120 °C with the peak temperature at 96 °C, which represents the phospholipid bilayer beginning to transform to a melted fluid crystalline stage at this temperature range and similarity related to the vaporization of bounded and free water molecules. The second endothermic event occurred between 173 and 222 °C with the peak temperature at 200 °C, which is attributed to the depolymerization of hemicellulose in the NFs, which is well known by the mass reduction of the glycoside linkage of the cellulose and BCP degradation. The third endothermic peak occurred between 266 and 288 °C with the peak temperature at 275 °C, which is attributed to cellulose degradation. The DSC results for Rephyll revealed that the stability of BCP is enhanced due to the incorporation of BCP onto the lipid bilayer with the anchoring effect of the NF through the NFW technology.

**Encapsulation Efficiency and Loading Capacity.** The encapsulation efficiency and loading capacity of BCP onto the phospholipids were investigated, which have registered good percentages, viz., 81.62 ± 3.52% and 13.62 ± 1.36%, respectively. These outcomes indicate that BCP can be effectively incorporated onto the lipid bilayer and maintain the powder form through the NF anchoring effect of the NFW technology.

**Stability Study.** The stability of the liposomal formulations, particularly in the powder form, is an important factor to determine its appropriateness for various applications, particularly in the functional foods and nutraceutical industries. For the period of 18 months of stability study period, no alteration was detected in the physical form and color of Rephyll. The content of BCP in Rephyll has been measured in different time intervals and is shown in Figure 7. The results demonstrate that there is a minor reduction in the content of BCP during the study period, which is not statistically significant and reveals the good stability of Rephyll.

**In Vitro Release Study.** The in vitro drug release profile of BCP and Rephyll is illustrated in Figure 8. Both the samples showed a two-phase release pattern by a quicker release in the primary phase and then a sustained release at a persistent rate, like that found in liposomal systems incorporating bioactive molecules.19,20 A rapid release of about 95% of BCP sample was witnessed in the first 8 h. The occurrence of rupture release was not found in the release curve of Rephyll and the results revealed a fast primary rupture until 12 h to about 81% and subsequently a slow and sustained release. This is due to the good encapsulation of BCP onto the liposomal core, reaching further the surface slowly and accordingly taking longer to be released, which leads to a slower release rate from Rephyll when related to the BCP sample.

**The Effects of Rephyll on DOMS in Humans.** A total of 20 eligible healthy male subjects participated with the mean age of 21.85 ± 2.91, mean height of 169.66 ± 2.93 cm, and mean weight of 60.65 ± 16.62 kg. Any serious adverse events did not occur throughout the entire study and treatment period. There were no protocol deviations reported throughout the complete study.

In this study, the effectiveness of Rephyll was assessed with various functional measures comprising pain VAS, isometric flexion strength by isometric MCV test score, muscle flexibility by relaxed arm angle degree, serum CK, and myoglobin and the results are given in Table 1. The isometric MCV test score was increased from 33.37 to 36.70 from baseline to the end of the study period in the Rephyll group, whereas a slight increase was registered in the placebo group (Table 1), suggesting the improvement of the isometric strength due to the treatment of Rephyll. Statistical analysis revealed a significant increase \((P = 0.01)\) in the isometric strength in Rephyll, confirming the beneficial effects of the encapsulated BCP on DOMS. However, there are no significant changes in the muscle flexibility.

The pain VAS score was significantly reduced from 3.55 to 2.50 in the Rephyll-treated subjects from baseline to the end of the study; in contradiction, the pain VAS score increased marginally from 3.25 to 3.75 in the placebo group, which is not statistically significant (Table 1). The pain VAS score of Rephyll was registered to statistically significant decrease within the group \((P = 0.0003)\) and intergroup \((P = 0.003)\) between Rephyll and placebo, which confirms the advantageous effect of Rephyll in the treatment of DOMS for pain relief.

CK did not show any statistically significant variations within the group as well as between the groups (Table 1). In the level of myoglobin, there are no significant changes in the Rephyll group, whereas the myoglobin levels gradually increased in the placebo group until the end of the study (Table 1), which support the clinical findings that a good safety profile indeed has a favorable effect and aids in the treatment of DOMS.

Various nutritional supplements have been showing the effectiveness in managing the DOMS due to their anti-inflammatory property. The markers of muscle damage after
### Table 1. Effect of Rephyll on Different Functional Measures of Muscle Damage and Following Exercise

| Parameter                        | Placebo (N = 20) | Rephyll (N = 20) |
|----------------------------------|------------------|------------------|
|                                | baseline day 1 mean ± SD | day 2 mean ± SD | day 3 mean ± SD | day 4 mean ± SD |
|                                | 31.57 ± 7.23      | 38.42 ± 8.19     | 33.97 ± 6.72     | 36.80 ± 6.19    |
|                                | 0.39              | 0.70             | 0.70             | 0.01            |
|                                |                  |                  |                  | 0.94            |
|                                |                  |                  |                  | 0.47            |
|                                |                  |                  |                  | 0.34            |
|                                |                  |                  |                  | 0.59            |
|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |
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|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |
|                                |                  |                  |                  | 0.09            |

The subjects in the Rephyll group experienced a significant reduction within the group and intergroup in pain VAS, which suggests that the consumption of Rephyll can reduce the DOMS induced by maximal voluntary contraction exercise. The subjects in the Rephyll group experienced a significant reduction within the group and intergroup in pain VAS, which suggests that the consumption of Rephyll can reduce the DOMS induced by maximal voluntary contraction exercise. The subjects in the Rephyll group experienced a significant reduction within the group and intergroup in pain VAS, which suggests that the consumption of Rephyll can reduce the DOMS induced by maximal voluntary contraction exercise.

Rephyll, the unique natural pain management formulation, incorporates the phytocannabinoid BCP, which is derived from black pepper, formulated using nanofiber weaving technology, commercially known as Zeal technology. The current study was also focused on assessing the influence of Rephyll on DOMS and indicators of muscle injury and destruction, pain, and inflammation. To the best of our knowledge, this was the first study to investigate the effects of BCP in a liposomal formulation to minimize the DOMS.

DOMS normally progresses between 24 and 48 h after a strange workout procedure that comprises eccentric muscle contractions. In this study, the VAS scale was increased in the first 2 days, proposing that the muscle injury and destruction had happened. However, the VAS scale score was significantly decreased after day 2 in the Rephyll group, which suggests that the consumption of Rephyll can reduce the DOMS induced by maximal voluntary contraction exercise. The subjects in the Rephyll group experienced a significant reduction within the group and intergroup in pain VAS, which strongly trended for favoring Rephyll for its improved and enhanced relief of DOMS pain due to the bioavailable form of BCP fabricated by the NFW technology.

The Rephyll group experienced considerable modifications in isometric flexion strength between pre-exercise intervention and the acute post-exercise time period. The exercise intervention is of anaerobic nature and is very stressful, which tempt oxidative stress, disrupts cellular membrane, and induce muscular and cellular damage linked with DOMS. Moreover, both groups registered considerable modifications in DOMS from pre-exercise, demonstrating that both groups experienced nearly the same perceived levels of muscle soreness, which was reconfirmed by elevated levels of serum CK and myoglobin and also assured that the exercise intervention selected is effective at making muscle soreness.

Rephyll was found to improve the isometric flexion strength and shows a positive trend in increasing the muscle flexibility. This can be due to the cannabimimetic anti-inflammatory activity of the bioavailable form of BCP containing Rephyll, which can be achieved by better return of interstitial fluid and cells to the bloodstream, consequently reducing edema and swelling and diminishing the development of prostaglandins and involvement of other eicosanoids in the inflammatory response to damage. These synergistic mechanisms of decrease in inflammation thus could diminish the pain-related DOMS.
Figure 9. Schematic illustration of the main signaling pathways and receptors through which Rephyll impacts anti-inflammatory and antioxidant activities. Blue arrows indicate pathways initiated by inflammatory response/DOMS, and red arrows indicate CB2 receptor-mediated mechanisms initiated by Rephyll. Continuous red lines indicate stimulation, and dotted red lines indicate inhibition. IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; CB2 receptor, cannabinoid receptor type 2; P13K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor kappa light chain enhancer of activated B cells; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TLR4, Toll-like receptor 4; MD-2, myeloid diκβ; TNF-α, tumor necrosis factor alpha; CB2 receptor, cannabinoid receptor type 2; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor kappa light chain enhancer of activated B cells; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TLR4, Toll-like receptor 4; MD-2, myeloid differentiation factor 2; CD14, cluster of differentiation 14; IKK, IκB kinase; JNK, c-Jun N-terminal kinase.

in addition to support in the recovery of strength after eccentric exercise.

In the modulation of acute and chronic pain, the endocannabinoid system has been revealed to play a considerable role and act a main healing target.33,44 Together, cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) have been revealed to contribute in inflammatory and pain, although the absence of central nervous system side effects sets onward the stimulation of CB2 receptor, a smart target for the management of pain, as established in well-authorized representations of severe pain, nociception, persistent inflammatory pain, post-operative pain, chemogenic pain, cancer pain, and neuropathic pain.10,12,45

BCP is the foremost natural CB2 receptor and a potent phytocannabinoid, which is described to have anti-inflammatoire activity, because this phytocannabinoid plays a significant role in pain variation, which may be accepted as a promise pain management. BCP performs as a discriminatory binder for the CB2 receptor, the therapeutic target for the management of inflammation, atherosclerosis, pain, osteoporosis, etc. It is also proven to have antioxidant, anticarcinogenic, and antimicrobial properties and is an important oral nutraceutical. BCP utilizes an anti-inflammatory effect in the carrageenan-induced edema test.12,46 Cho et al. demonstrated that BCP improved dextran sulfate sodium-induced colonic inflammation in BALB/c mice by regulating the proinflammatory cytokine interleukin 6.11 Similarly, BCP prevents inflammation and tissue damage in colitis and nephrotoxicity models in a CB2 receptor-dependent manner.12,14

The CB2 receptor is extensively found in peripheral tissues, particularly in immune cells, and its activity can stimulate antinociception by the direct or indirect stimulation of the final pain variation pathway.51,52 Consequently, selective agonists of CB2 receptors, particularly BCP, have been recommended to encourage the release of endogenous β-endorphin peptide precursors from keratinocytes, leading to the activation of opioid receptors in primary afferent neurons and, subsequently, analgesia.53 Otherwise, the analgesic effects may be due to the direct stimulation of CB2 receptors in peripheral neurons54 or even in brain regions related to pain modulation, for instance, the thalamus, cerebellum, and brainstem.49,55–57

BCP was established to prompt a complete agonist action on CB2 receptors, G-protein-combined receptors demonstrating a significant healing target in several illnesses. Stimulation of CB2 receptors remarkably performed devoid of psychotropic adversative outcome of cannabinoid conflict to the CB1 receptors. Additionally, it stimulates peroxisome-proliferated activator receptors, obstructs pathways generated by the stimulation of Toll-like receptor complex CD14/TLR4/MD2, diminishes immune inflammatory progressions, and reveals synergy with μ-opioid receptor-dependent pathways. Furthermore, it was found to be an effective antagonist of the homomeric nicotinic acetylcholine receptor (α7-nAChRs) and was devoid of effects facilitated by serotonergic and GABAergic receptors. It also modifies various molecular targets by varying their gene expression, signaling pathways, or complete direct communication. Several pharmacological activities such as anti-inflammatory, neuroprotective, immune-modulator, nephrprotective, cardioprotective, hepatoprotective, gastroprotective, antioxidant, and antimicrobial activities have been described in investigational studies. It provides effective healing assurance in neuropathic pain and neurodegenerative and metabolic diseases.55

The possible mechanism of action of the Rephyll bioavailable form of BCP was recommended based on the outcomes and recent studies, which suggest that the pain-relieving outcome may be a result of the interaction of the glutamatergic pathway by the way of the opioid system and with l-arginine/nitric oxide.49,50 The further probable mechanisms of action for BCP include variation of pain through the endocannabinoid pathways, the hindrance of the NF-κB pathway, and a decrease in cyclooxygenase-2 expression.
(COX-2) or tumor necrosis factor-α (TNF-α) and prostaglandin E₂ (PGE₂) release.⁵⁸,⁵⁹ Concisely, agonist action on CB2 receptors and inhibitor action on the Toll-like receptor complex, cluster of differentiation 14/Toll-like receptor 4/myeloid differentiation factor 2 (CD14/TLR4/MD2)-triggered pathways, and significant down-regulation of inducible nitric oxide synthase (iNOS) and COX-2 and reduction of proinflammatory cytokines (Figure 9) thus attribute to the anti-inflammatory properties of BCP.⁷ From the results of this study, both the systems may be involved in the severe and prolonged antinociceptive activity of BCP. In this study, consumption of Rephyll did not register any adverse events in the entire study, which confirms that it can be safely used by humans for relief of DOMS.

In summary, these results indicated, in agreement with the majority of the studies in the literature, that the Rephyll group confirmed improved isometric forearm flexion strength values than the placebo group, as well as higher reduction in subjective pain rating in nearly identical baseline values of the previous eccentric exercise protocol. After the eccentric exercise, there were no changes between the study groups in the patterns for relaxed arm angle and plasma creatine kinase activity, although myoglobin concentration showed a decrease in comparison with placebo. These outcomes delivered primary confirmation that the Rephyll supplement is indeed beneficial for decreasing strength loss immediately after the eccentric exercise and for assisting in the short-term (24–72 h) recovery of strength after acute upper arm-induced DOMS. Thus, Rephyll has the potential for inhibiting DOMS, as recommended by its effects on pain intensity and muscle damage without any adverse effects. Larger studies with more refined study tools can be employed in the prospective studies to confirm these outcomes and additionally elucidate the mechanism of action.

To conclude, a novel powder form of liposomal β-caryophyllene (Rephyll) formulation was designed and developed by nanofiber weaving (NFW) technology for enhanced bioavailability and improved stability of β-caryophyllene (BCP). TEM images clearly indicate that Rephyll in solution is nanosized, has a smooth surface, and is spherical-shaped without aggregation, demonstrating the good encapsulation of BCP onto the phospholipid bilayer, and stabilizes through the anchoring effect of nanofiber by the NFW technology, which is further confirmed by the FT-IR and DSC analyses. The encapsulation efficiency, loading capacity, stability, and in vitro drug release studies revealed that Rephyll is a good delivery system for BCP. The effects of Rephyll were evaluated by a randomized, double-blinded, crossover-designed, placebo-controlled study. The oral consumption of Rephyll significantly reduced the VAS pain score, which revealed that Rephyll has a potential for preventing DOMS. Furthermore, the improved recovery on pain intensity and muscle injury without any side effects revealed that the product Rephyll can be an alternative supplement for pain management.

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**Notes**

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