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The effects of supplementing with sperm and prenatal deprivation on the neuropsychological development of Rat F1 offsprings

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Abstract:

Research on protein malnutrition, also known as protein-calorie malnutrition, is growing in popularity because it affects the brain's structural proteins, enzymes, and neurotransmitters. The mother's foetus may be born with developmental defects if she consumes insufficient amounts of protein. Therefore, a major contributing factor to perinatal death and morbidity is poor nutrition for the expecting mother. Eating habits and mental health are strongly correlated, with recent research emphasising the linear nature of this association. Our most recent research found that kids born to low-protein moms had low anxiety, poor habituation, delayed reflex ontogeny, inadequate forelimb neuromuscular strength, and impaired cognitive performance. PMN, or protein malnutrition, is a worldwide issue that mostly impacts people in Asia and Africa. It negatively affects both the physiological and structural elements of the hippocampus circuitry. Despite growing evidence that PMN promotes alterations in the neurological system, very little is known about the effects of maternal nutritional supplements on neurons and glial cells during malnutrition. In the hippocampus of F1 offspring, we sought to examine the protective effects of maternal supplementation with Spirulina against PMN-induced oxidative stress, reactive gliosis, and neuronal damage. Fifteen days before conception, twenty-four healthy, three-month-old Sprague Dawley females (n = 24) were placed on diets high in protein (LP; 8% protein) and low in protein (NC; 20% protein). The low protein group (LP SPI) and the normal control group (NC SPI) were created from the amount of sperulina supplementation (400 mg/kg/b.wt. orally throughout the gestation and lactation period) given to the females in the NC and LP groups. F1 progeny births were employed in

this investigation. The present study integrates neurochemical and morphometric evaluation of glial cells and neurons, building on previous findings of improved neurobehavioral and cognitive skills in protein-deprived rats supplemented with spirulina. Consequently, some of the neuropathological alterations associated with PMN were partly avoided. Reactive gliosis and apoptotic cell population were reduced, oxidative brain damage was attenuated, dendritic branch complexity was increased with fewer damaged neurons, and the density of mushroom-shaped spines was increased. The findings imply that cellular alterations brought on by PMN in the hippocampal regions may be partly undone by moms taking supplements of spirulina, which raises the prospect of using spirulina in treatments for malnutrition. Recurrent exposure to various early-life stresses is expected to have an impact on a person's behavioural development and raise their risk of developing neuropsychiatric illnesses. The goal of the current research is to investigate behavioural impairments between adolescence and adulthood by simulating such conditions in a rat model. A low protein (LP; 8% protein) or control (20% protein) diet was fed to female Wistar rats (n = 32; 140–150 gm) 15 days before to conception, and the diet was followed throughout the trial. A total of eight experimental groups were created by intraperitoneal injection of deltamethrin (DLT—pyrethroid insecticide; 0.7 mg/kg body weight; PND 1 to 7) or lipopolysaccharide (LPS—bacterial endotoxin; 0.3 mg/kg body weight; PND 3 and 5) or DLT+LPS (LP—pyrethroid insecticide; 0.7 mg/kg body weight; PND 1 to 7). At one, three, and six months of age, F1 rats were subjected to several neurobehavioral examinations, including the rotarod, elevated plus maze, open field, light and dark box tests, and others. Age-matched control rats and LP rats were examined, and it was shown that the former were much more vulnerable to one or more exposures. They also exhibited very severe behavioural abnormalities, such as attention impairments, hyperactivity, and low anxiety, all of which are suggestive of neuropsychiatric disorders including schizophrenia and ADHD. This indicates that early childhood exposure to several strikes may increase an individual's likelihood of developing abnormalities.

1 INTRODUCTION

Fruits and vegetables are grown in large numbers in developing nations like

ISSN: 2057-5688

Bangladesh and India, but much of the fruits are wasted because there is not enough cold storage space or suitable transportation. Small businesses that deal with food also have a number of restrictions when it comes to preservation and marketing. The food and beverage industry are attempting to deliver both low-cost potential preservative and raw ingredients as a result. Preservatives work well to extend food's shelf life and stop microbial development that causes spoiling. Due to some of the deterioration, the food, pharmaceutical, and meat sectors have used a vast array of artificially synthesised preservatives.

However, breathing problems, nasal congestion, sneezing, skin rashes, wheezing, and even a severe anaphylactic response may all be brought on by the chemical preservative. On the other hand, food deterioration and loss of nutritional content may be minimised by chemical food preservatives (Emamifar et al., 2011). For health reasons, individuals choose to utilise natural food preservatives. According to Lombardo et al. (2019), nanomaterials such as metal nanoparticles, carbon nanotubes, nanodots, and other active nanocompounds may be used to create biosensors for the creation of a new class of antimicrobial drugs that are effective against a wide range of microbes.

The materials extracted from Spirulina platensis are used as preservatives; however, synthetic nano dots made from Spirulina extract have special qualities such as a large surface area/volume ratio, unique size, heat conductivity, magnetic properties, and some catalytic and antimicrobial activity, which make them an improved food preservative (Rai et al., 2012). In addition, nanoparticles produced from Spirulina platensis have both antioxidant and antibacterial properties. A small number of studies have shown the antioxidant qualities of caffeine, which include its ability to scavenge free radicals and provide cytoprotection against UV and radiowave exposure (Sato et al., 2011).

Therefore, caffeine acid has the potential to be utilised in fruit juices as a

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substitute for regularly used synthetic preservatives. Thus, spirulina's caffeic acid-rich antioxidant extract has several health advantages and may be utilised as both a potent antioxidant and a food preservative. To guarantee the highest concentration of antioxidant-rich extract, the extraction process may be optimised under ideal circumstances. Therefore, it may be successfully marketed as a natural preservative with nutraceutical qualities if the purified antioxidant-rich extract is used in lieu of the synthetic preservative.

It is often known that indigenous people from many regions of the globe use spirulina as food. The existence of colours, nutritional benefits, and therapeutic qualities have drawn scientists and people from all over the globe to this cyanobacterium in recent years. These days, this organism is sold as a dietary supplement and may be found in cakes, biscuits, noodles, tablets, capsules, and other forms of food (Lee, 2017). It is used in cakes, nankhatai, ladoos, and pakoras in India. A number of nations are creating strategic plans for the cultivation and use of spirulina. The spirulina market is expanding quickly in China. Nearly 350 tonnes of dry powder, used for food, feed, and medication, are produced by more than 80 manufacturers, according to data from 2007 and 2018 (Lee, 2007; Tseng, 2011).

The mid-1960s saw the rediscovery of spirulina. A blue-green cake that was being sold in the food market of Fort Lamy, Chad, was reported by French botanist Jean Leonard on an expedition to Africa. The Kanembu tribe, who live among the alkaline lakes of Chad and Niger, ate this cake, known as "Dihe" in the local tongue. When the Spanish invaded Mexico in the sixteenth century, they found that the Aztecs who lived in the valley of Mexico's capital, Tenochtitlan (modern-day Mexico City), were consuming a blue-green cake that the natives named "tecuitlatr," which they had collected from the lake. This was used in a manner reminiscent to the Kanembu tribe's 'Dihe'. It is evident that this diet comprised a blue-green alga, even if the precise composition of the food is unknown. Spirulina maxima is now the most common species in those waters.

ISSN: 2057-5688

Planktonic cyanobacterium Spirulina maxima and Spirulina platensis are the two traditional species of spirulina; the former is found in Africa, Asia, and South America, while the latter is essentially limited to Central America. Spirulina forms massive populations in tropical and subtropical water bodies characterised by high levels of carbonate, bicarbonate, and pH.

2 LITERATURE SURVEY

In prokaryotes, two component signalling systems function as redox sensors. They typically consist of a histidine kinase that senses the signal and a response regulator that functions as a transcription factor. The transmem brane sensory kinase auto phosphoryl ates a histidine residue in response to the presence or absence of an external stimulus. The phosphoryl group is then transferred from the histidine to an aspartate residue in the response regulator. The induced conformational change in the response regulator changes its DNA binding affinity and so promotes gene expression. A number of components that may be involved in signal transduction have been identified in recent studies. These signal transduction pathways and the various sensors are integrated into complex pathways in animals and plants.

Plants and Cyanobacteria have defence mechanisms against oxidants.

Because they produce oxygen during photosynthesis and consume it during respiration, cyanobacteria and higher plants may be more susceptible to oxidative stress than other prokaryotes and eukaryotes. To prevent oxidative damage caused by excessive light, cyanobacteria and higher plants have developed a variety of defence mechanisms, including antioxidant enzymes, non-enzymatic cell components, and short- and long-term regulatory responses, which involve changing the structure of the photosynthetic apparatus.

Long-term responses involve changes in the expression of genes encoding proteins related to photosynthesis. This regulation serves to replace the damaged proteins during light stress and to adapt photosystems to different light conditions. These short-term processes are well characterised in algae and

ISSN: 2057-5688

higher plants, but they are not yet well characterised in cyanobacteria because they contain phycobilisomes as PS II antenna.

Light regulates the expression of these genes via redox sensory mechanisms in this organism. Ferredoxin binding protein from photosystem I encoded by ps dE and D1 protein in photosystem I encoded by psbA have both been studied. Blue and far-red lights induce the expression of psbA transcripts, and orange light induces accumulation of psaE transcripts (Bissati and Kirilovsky, 2001). Multiple light- and redox-sensing systems in plants, and synechococystis PCC6803 genes show similarities with these regulatory genes. Therefore, regulation of their expression may be similar to that of plants.

Plants and cyanobacteria's enzymatic and nonenzymatic antioxidant defence:

Plants and cyanobacteria contain antioxidant defence systems that can be either enzymatic or nonenzymatic. These defence systems are involved in scayenging different reactive oxygen species and are present in intracellular compartments as well as, to a limited extent, in the apoplast. The regulation of the antioxidant defence system in plants is well-established, and among cyanobacteria, various mechanisms have been reported in different organisms.

Protection against oxygen sinelet:

Superoxide radicals are scayedenged by the metalloenzymes, superoxide dismut ases. This family of metalloenzymes catalyses the disproportionation of superoxide to molecular oxygen and H2O2. Since it remoyes the superoxide, it decreases the chance of hydroxyl radical formation from superoxide by the metal catalysed Haber-Weiss type reaction. The existence of multiple forms of SOD may be due to the localization of superoxide formation in the membranes as well.

Superoxides are negatively charged and unable to pass through the phopholipid bilayer, which effectively traps them within the membranes. In plants and cyanobacteria, superoxides are formed in both the cytosol and the thylakoid membranes. Superoxide radicals formed within membranes are scavenged by membrane-bound SODs, which are

ISSN: 2057-5688

categorised into different classes based on their metal cofactor. MnSODs are found in the cytosol of both eubacteria and cyanobacteria. FeSODs are present in the cytosol of both eubacteria and cyanobacteria as well as in the chloroplast stroma of plant cells. Various SODs have specific protective roles within the cell. FeSOD shields the cell against V'• superoxides that are generated in the cytosol and are generated from electron transpor

Cytosolic SOD is not involved in the defence of cell components against superoxide generated within thylakoid membranes. This defence may be attributed to MnSOD, although research using MnSOD-deficient mutants is necessary to verify its potential contribution (Thomas et al, 2008; Arora et al, 2002).

Hydrogen peroxide scavenging:

Two types of enzymes, catalase and peroxidases, are typically responsible for removing hydrogen peroxide from aerobes. Catalases directly catalyse the breakdown of H2O2 into ground state O2, and they are haem-containing enzymes that are sensitive to azide, cyanide, and HOCl. Some bacteria, however, exhibit the presence of a pseudocatalase, which lacks haem and is therefore insensitive to azide or cyanide. This enzyme contains Mn (III) ion.

Welinder (2002) divided plant-type peroxidases into three structurally unrelated classes: class I includes bacterial peroxidase, yeast cytochrome c peroxidase, and ascorbate peroxidase; class II includes fungal peroxidases like lignin and manganese peroxidase; and class III includes classical plant peroxidases like horseradish peroxidase. Peroxidases remove H2O2 by coupling its reduction to H2O2 with oxidation of another substrate.

All aerobic organisms contain catalases; plants have multiple catalases encoded by multiple genes; since catalase only exists in leaf cell peroxisomes, a peroxidase reaction uses ascorbate as an electron donor to scavenge the H2O2 produced in chloroplasts. Certain catalases are also capable of performing specific peroxidase type reactions.

ISSN: 2057-5688

Ascorbate peroxidase and ascorbic acid:

Ascorbate peroxidase (APX) has been found in the cytosol, mitochondria, and chloroplasts. In the chloroplast, APX and SOD are found in both soluble and thylakoid-bound forms. SOD uses superoxide produced at the membrane surface to produce H2O2, which APX then scavenges. Ascorbate peroxidases are haem proteins that differ from class III peroxidase in that they have a higher affinity for ascorbate as reducing substrate. Salicylic acid and thiol reagents are known to inhibit APX. In the absence of ascorbate, ascorbate peroxidase in the chloroplast can become inactive. In plants, seven isoforms of the enzymes have been identified, each with distinct cellular locations and structural traits.

3 METHODOLOGY

The methodology was shown with reference to Wetchakul et al.'s modified test (2019). The reduction of colourless tripyridyltriazine ferric complex (Fe3+) to blue-colored tripyridyltriazine ferrous complex (Fe2+) provides the foundation for this test. Ten volumes of 300 (mM) acetate buffer (pH 3.6), one volume of 20 (mM) ferric chloride, and one volume of 10 (mM) TPTZ (2,4,6-tri (2-pyridyl)-s-triazine) in 40 (mM) hydrochloric acid were combined to create the FRAP reagent. All required solutions were made fresh before to use. Different amounts of ferric chloride (FeCl3) were used to make the standard solution. Following that, 100 µL of standard and extract solution were combined with 3 mL of newly prepared FRAP reagent. The solution was maintained at 37 °C for 30 minutes in a water bath. UV-Vis spectroscopy was used to measure the optical density at 593 nm after the recommended incubation time. Using a linear FeSO4 equation expressed as mM FeSO4 equivalent/g of dried algae, the FRAP value was determined. Every experiment was run three times.

Calculating the flavonoid content

Aluminium chloride (AlCl3) was used to measure the total flavonoid content of the UME extract using a slightly modified version of Nabavi et al.'s (2008) methodology. The remaining process was the same as previously stated.

Tandem mass spectrometers with liquid chromatography (LC-MS-MS):

According to Terpinc et al. (2016), the extracted UME was coded as UME best

ISSN: 2057-5688

while operating under ideal conditions. To identify the phenolic components in the extract, the sample was subjected to LC-MS/MS (Waters 2695 Separation Module PDA, with Mass-Lynx 4.1, Quattro mini -TM API, USA). Before being injected into the LCMS, the UME best extract was first diluted in 0.1% formic acid and filtered through a 0.45 micron filter (Durapore, Billerica, USA). Following that, a microsyringe was used to inject the filtrate sample onto a C18 analytical column. In the column, the inject volume was 20 µL. The required sample was eluted from the column using gradient mobile phases A (HPLC grade distilled water and 0.1% formic acid) and B (HPLC grade methanol and 0.1% formic acid). The mobile phase's flow rate was set at 1 mL/min. In the m/z range of 100–1200, the mass spectra using the negative ESI ionisation mode were used.

LC-MS analysis of the UME best extract to determine the phenolic chemicals present.

The desolvation curved line's voltage and temperature were set at +85 V and 230 °C, respectively. Likewise, the nitrogen nebulizer gas and probe voltage gradient flow rates were set at +4.5 kV and 4.5 L/min, respectively.

Calculating the amount of caffeic acid in the best UME extract produced using high performance liquid chromatography (HPLC) under ideal extraction conditions:

The methodology of Mbous et al. (2017) was followed in the estimation of caffeic acid. Before being injected into the HPLC, the UME best extract was diluted in mobile phase solution and filtered through a 0.45 micron filter (Durapore, Billerica, USA). The isolation of HPLC (1300 series Agilent ZORBAX SB Technologies Inc., Alpharetta, GA, USA) was assessed using a C18 column that had a tandem connection and a particle size of 5 µ m and measured 150 × 4.6 mm.

Spirulina extract analysis using high performance liquid chromatography (HPLC).

Starting at 0% – 40% B for 10 min, 40% – 100% B for 10 min, 100% B for 5 min, and 100% – 5% B for 5 min, the gradient mobile phase solution comprising water and acetic acid in the ratio of 95:5 (A) and methanol, acetonitrile, and acetic acid in the ratio

ISSN: 2057-5688

of 95:5:1 (B) was utilised. The mobile phase's flow rate was set at one millilitre per minute. A 20 µL inject volume of sample was used in the column. It was ambient temperature in the column. Three injections of each solution were made into the column. Mobile phase B was used to dissolve the standard caffeic acid (HPLC grade, Sigma Aldrich, Kolkata, India), and various concentrations of the standard solution (20, 40, 60, 80, and 100 mg/mL) were prepared in order to build a calibration curve. Using a linear equation, the quantification of caffeic acid in microalgae samples was determined as µg/g of dried microalgae, taking into account the mean peak areas.

Microalgae sample supercritical fluid extraction (SCFE):

Using an Applied Separations Spe-ed SFETM equipment, supercritical fluid extraction was performed at Lehigh, USA. A precise weight of fifty grammes of freeze-dried Spirulina biomass was determined. Next, a 100 mL stainless steel jar was filled with the sample. To prevent leakage from the jar, cotton wool needed to be placed at both ends. Using a micro-meter valve, pressurised CO2 was supplied from the bottom section of the steel tank at a rate of 20 L/h. A co-solvent pump was also used to apply 100% ethanol as a co-solvent from the bottom end at a rate of 1

The whole extraction period was scheduled for 120 minutes in accordance with the experimental setup, and this was followed by a static and dynamic time. The extracts were collected in 30 mL SFE glass vials after the static time. To prevent glass vials from cracking, the outflow valve temperature was set higher than the oven temperature. The resulting microalgae extract is weighed using a gravimetric scale, and it is thereafter kept inert nitrogen gas in hermetic screw-capped glass containers that are tinted amber until further examination at 4 °C. Using a Box-Behnken design (BBD) with three tiers of independent parameters, the SCF extraction parameters were optimised.

Data analysis, model creation, and experimental design were all done using the programme Design Expert. For the extraction trials, the three independent variables—pressure (X1), extraction temperature (X2), and static time (X3)—as well as the dependent variable—

ISSN: 2057-5688

caffeic acid content—were selected. Three variables were used in seventeen tests carried out in accordance with BBD, and four trials at central locations were arranged in a randomised sequence to assist reduce unexplained variability in the response that was produced. Each independent factor's bounds were set based on the results of earlier pilot studies and literature reviews. Three levels of extraction pressure (200–400 bar), extraction temperature (30–50 o C), and static durations (30–90 min) were determined based on the preliminary studies.

Calculating total polyphenol content:

With a slightly modified version of Abdul et al.'s method, the total polyphenol content of the SCFE microalgae extracts was determined using the Folin–Ciocalteu reagent (FCR) (2017). In an ethanol solution, various concentrations of supercritical fluid extract were produced. The remaining procedure was comparable to the previously stated one.

4 RESULTS

Trials were conducted in order to ascertain the extract's effectiveness against E. B. Coli. S. subtilis. Cholorae and V. aureus. The conventional technique of disc diffusion on nutrient agar plate was used to conduct the in vitro antibacterial experiment (Rahman et al., 2013). On nutrient agar slants, stock cultures of bacterial strains were inoculated for cold storage. By moving the stock culture of the bacterial strains to the nutrient broth, the inoculum was created. The petriplates and nutrient agar medium were autoclaved for 15 minutes at 121°C and 15 lb/inch2 of pressure. Subsequently, the heated medium was transferred onto the sterile petriplates and let to stand for a full hour. Next, using a spreader, the bacterial inoculums were evenly distributed across the nutritional agar. The cork borer on the petriplates created the bore. After that, various extract concentrations were added to the bore and let five minutes to diffuse. The petriplates were then placed in a BOD incubator and incubated for 24 hours at 37 °C. The zone of inhibition developed after a certain amount of incubation time, and the zone was measured in millimetres using a clear ruler. Every test was run in triplicate.

Active compound isolation and purification using thin-layer chromatography (TLC):

ISSN: 2057-5688

To separate and separate the active ingredient from the microalgae, the SCFE extract was put through a TLC plate. A flowing solvent solution with a ratio of 1.5:6:2.5 for acetic acid, butanol, and water was used to separate the refined chemical from microalgae. Using a microneedle, an aliquot of SFE extract (10 µL) was spotted on a TLC plate. The areas were then given time to dry. The plate was then placed inside the TLC chamber with the solvent that was flowing. The TLC plate was taken out of the solvent and dried in a hot air oven at 45 °C after the solvent was contacted, around 1 cm from the top. Photographs were taken of the red spots in the TLC plate as they developed. To extract the pure active ingredient from the Spirulina extract, these coloured spots were removed from the TLC plate and dissolved with ethyl acetate.

Description of the separated substance:

Venkatesan et al. (2012) have showed that a certain technique has been followed in the FT-IR research. To create a clean, translucent disc, the separated material was combined in a mortar and pestle with dry potassium bromide pellets (KBr). The remaining process was the same as previously stated.

Analysis using a transmission electron microscope (TEM):

On a Cu-grid, the microalgae extract was drop-cast at a concentration of mg/mL. Following the instructions previously provided by Mahata et al. (2017), the samples were immediately examined under a TEM once the grid had dried at room temperature. Using the JEOL JEM 2100 apparatus, the HR-TEM was used to examine the microalgae samples and take pictures.

Analysis using nuclear magnetic resonance (NMR) spectroscopy:

Because it is only sensitive to the probing nuclei and can gather data on the physical characteristics at the atomic level, the NMR approach is a somewhat useful tool for studying nanoparticles (Son and Jang, 2013). One common design for NMR analysis is to use fixed nuclides of either 1H or 13C. Hydroperoxy groups and the hydrogen, carbonyl, and carbon atoms linked to conjugated-dieoic complexes may all be detected using 1H NMR.

1H has a chemical shift scale of just 14 ppm, whereas 13C has a chemical shift scale of 200 ppm. In an NMR tube

ISSN: 2057-5688

containing tetra methyl silane (TMS) as an internal standard, the extract was immediately combined with D2O. A DPX200 Bruker is used to capture the data (200 MHz for 1H and 500 MHz for 13C). All signals were compared to the reference solution within ± 0.1 ppm. Following baseline correction, all 1H and 13C NMR spectra were included, and a mean of three values was used for each computation.

Assessment of the capacity for auto-aggregation:

Using nutrient broth medium, the capacity of bacteria to auto-aggregate was assessed (Nikolic et al., 2010). An aliquot of extract was combined with the LB broth used to cultivate the Vibrio cholerae bacterium strain. The culture broth was then cultured in a shaker incubator for 48 hours at 120 rpm and 37 °C.

By measuring the optical density (OD) at 600 nm wavelength (OD600) using a UV-visible spectrophotometer (VARIAN, INC. CarryR 50 Bio, Australia), the bacterial growth was expected. Without centrifugation, an aliquot of 1.5 mL cell suspensions was taken in order to determine the OD at 600 nm. The remaining 1.5 mL aliquots were taken out and centrifuged at 5000×g for 10 minutes. Following the separation of the topmost layer, the absorbance was measured at 600 nm. Every test was run three times, and the average result was utilised in the computation. An aggregate index computation was made.

5 CONCLUSION

The antibacterial activity of the purified chemical was assessed using the standard technique of disc diffusion on nutrient agar plate against both Gram-positive and Gram-negative bacteria, in accordance with the Clinical and Laboratory Standards Institute (Gefen et al., 2017). The remaining process was the same as previously stated. The British Soft Drinks Association suggested certain common components, such as acids, colouring, sugar or sweeteners, preservatives, and health-promoting compounds like vitamins and minerals, for the creation of any basic soft drink. Ripe litchi was gathered for this investigation from a nearby market. Litchi was cleaned well and then peeled. To make litchi pulp, the litchi was pulverised in a food-grade mixer. Muslin fabric was used to separate the litchi pulp from the litchi liquid. After

ISSN: 2057-5688

filtering, the juice was cooked over low heat and 2% sugar was added. Subsequently, the juice underwent pasteurisation. Utilising Spirulina platensis nanodots and an antioxidant extract high in caffeic acid in litchi juice Following pasteurisation, 0.1 g of synthesised nanodot and 0.1 g of produced antioxidant-rich SCFE microalgae extract were added to the litchi juice to function as a preservative. RTS litchi juice (200 mL) was treated and then put into pre-sterilized glass bottles in a laminar air flow hood (Klenzaids LAF, Mumbai, India) whilst sterilised. A hand sealer was used right away to seal the glass bottles. After carefully cleaning and rinsing the glass bottles, they were autoclaved for 15 minutes at 121 °C. Ultimately, two groups of litchi juice—one treated and the other without extract—were exposed to various environmental factors. Beverage A, B, and C were the names given to the control juice, SFE-treated, and synthesised nanodot-treated litchi juice samples, respectively. Two sets of investigations were conducted with different storage temperatures, namely. both ambient (room temperature) and chilled (4±2 oC) at predetermined intervals (0th, 7th, 14th, 30th, 60th, 90th days).

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