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BIOLOGICAL EVALUATION OF ALPHA-TOCOPHEROL-CHITOSAN BIO-COMPOSITE: ANTI-INFLAMMATORY, ANTIOXIDANT, AND TOXICOLOGICAL ASSESSMENTS USING ZEBRAFISH EMBRYOS

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ABSTRACT

Objective: This study aimed to synthesize and evaluate a novel eco-friendly α-tocopherol–chitosan (CS) bio-composite for potential medicinal applications, emphasizing its thrombolytic, cytotoxic, anti-inflammatory, and antioxidant properties.

Methods: The α-tocopherol-CS bio-composite was synthesized and initially confirmed by color changes and visual observation. CS and the resulting bio-composite were characterized by ultraviolet-visible (UV-Vis) spectroscopy, which showed distinct peaks at 362 and 350 nm. Anti-inflammatory activity was assessed using bovine serum albumin, egg albumin, and membrane stabilization assays at 10–50 µg/mL concentrations. Antioxidant activity was analyzed statistically using two-way Analysis of Variance. Cytotoxicity was evaluated by the brine shrimp lethality assay, while anticoagulant and thrombolytic properties were measured by clot lysis at 50 µg/mL.

Results: UV-Vis spectroscopy validated the formation of the α-tocopherol-CS nanoformulation. The bio-composite demonstrated inhibition rates of 41–76% (bovine serum albumin), 50–74% (egg albumin), and the highest inhibition of 52–84% and an IC_{50} value of 5 µg/mL (membrane stabilization). Antioxidant activity was highly significant (time, $p<0.0001$; column factor, $p=0.0058$; time-treatment interaction, $p=0.0024$; subject variability, $p=0.0455$). Clot lysis occurred within 8 min at 50 µg/mL, indicating enhanced anticoagulant and thrombolytic effects. Cytotoxicity was markedly reduced at higher extract concentrations (40 and 80 µg/mL, $p < 0.01$). Overall, the bio-composite exhibited potent antioxidant and anti-inflammatory activity comparable to ascorbic acid.

Conclusion: The biologically synthesized α-tocopherol-CS bio-composite shows promising anti-inflammatory, anticoagulant, thrombolytic, antioxidant, and reduced cytotoxic properties. These findings highlight its therapeutic potential and suitability for further nanomedicine research and development.

Keywords: Chitosan, α-Tocopherol, Anticoagulant, Anti-inflammatory, Antioxidant, Embryonic toxicity.

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INTRODUCTION

Inflammation is a physiological reaction of the immune system that aids in the identification and removal of damaging stimuli while also initiating the process of tissue repair and healing. Prolonged inflammation that lasts for several months to years is also known as chronic inflammation [1,2]. Chronic inflammation has a major role in the growth of long-term disorders, like tumors and cardiovascular disease. Its prevalence in emerging nations has been rising recently. Numerous studies point to a significant role for inflammation in all stages of atherosclerosis [3]. The key players in atherosclerosis include T lymphocytes, monocytes-macrophages, cytokines, chemokines, and cell adhesion molecules. Apart from being a cardiovascular disease (CVD) risk factor, C-reactive protein (CRP) is a major biomarker of inflammation that may also be involved in the development of atherosclerosis [3,4]. The administration of alpha-tocopherol (α-tocopherol), particularly at high dosages, has demonstrated a reduction in the pro-inflammatory release of cytokines [5], including interleukin-1β, interleukin-6, and tumor necrosis factor-α, as well as the chemokine interleukin-8, and the suppression of monocyte adhesion to endothelial cells [6]. Moreover, it has been demonstrated that α-tocopherol lowers CRP levels in individuals with CVD and associated risk factors (such as diabetes and smoking). In addition, studies have demonstrated a reduction in interleukin levels that promote inflammation and concentrations of plasminogen activator inhibitor-1, upon α-tocopherol supplementation *in vivo* [7]. The development of innovative anti-inflammatory medications is continuously of interest to restrict the chronic inflammation processes in the human body, which include osteoarthritis, colitis, skin irritation, neurological diseases, and cancer [1,47]. Since the anti-allergic treatments to relieve inflammation are generally both corticosteroids and non-steroidal drugs for inflammation (NSAIDs), which create a lot of undesirable drug responses, including stomach itching, and liver and kidney problems on long-term consumption [8].

NSAIDs primarily suppress cyclooxygenase (COX) enzymes. COX enzymes are responsible for converting arachidonic acid into thromboxanes, prostaglandins (PGE2), and prostacyclins. NSAIDs are beneficial due to eicosanoid deficiency. Thromboxanes promote platelet adhesion, while PGE2 regulates vasodilation, hypothalamic temperature, and antinociception. COX enzymes in practically every human tissue produce PGE2, which cause inflammation through vasodilation [3]. PGE2 is produced from arachidonic acid by the COX enzyme's two isoforms, COX-1 and COX-2. NSAIDs lower the level of PGE2 in the body by inhibiting COX activity. As a result, pain, heat, and inflammation are reduced [48].

Mitochondrial metabolism promotes tumorigenesis by producing reactive oxygen species (ROS) during oncogenic change and the development of cancer. An increase in ROS production, the cellular components suffer structural damage, leading to cancer and inflammation, and other diseases [9]. In recent years, there has been a trend to investigate natural compounds that have considerable antioxidant activity, which can influence the redox reactions occurring in a cell and can inhibit and modulate free radical-mediated reactions [10]. In many of these plants, bioactive substances have shown promise as treatments for a variety of chronic illnesses, such as cancer, heart disease, and neurological problems. This therapeutic effectiveness is frequently attributed to their diverse biological activities, which include antiviral, antifungal, anticancer, antioxidant, and macrophage properties [11]. They also show the ability to alter cellular activity in cells linked to inflammation, such as mast cells, neutrophils, lymphocytes, and macrophages. Known as hyperaccumulators, some plants exhibit the phenomenon of metal accumulation to considerable degrees, making them appropriate for use in metal extraction processes [12]. Several therapeutic compounds have been delivered using natural polymers, particularly polysaccharides. After cellulose, the most common natural polymer is chitosan (CS), which is produced by partially deacetylated chitin, which is present in many fungi and invertebrates such as insects, prawns, and crabs [13].

Recently, considering polymeric nanoparticles have medicinal benefits, their development as drug delivery vehicles has gained attention. Polymeric nanoparticles have the potential to shield the medicinal component from deterioration, guaranteeing the drug's durability and improving cellular availability for increased effectiveness of the entrapment active component [14,15]. When it comes to biomedical applications, biocompatibility of biomaterials is essential since it guarantees that there won't be any unfavorable responses when they come into contact with biological systems. Because of its structural resemblance to glycosaminoglycans, CS has intrinsic biocompatibility and promotes tissue regeneration, cellular adhesion, and proliferation [16]. In addition, α-tocopherol, a naturally occurring antioxidant, helps to lessen cytotoxicity brought on by oxidative stress, which improves the biocompatibility of materials based on CS [17]. Being a naturally occurring biodegradable, environmentally benign, biocompatible, and non-toxic substance [46] that is acceptable for human ingestion, it is a powerful chemical of interest in many different sectors. It is used for food packaging and drug delivery as it significantly enhances the antibacterial and antioxidant properties [18]. In addition, CS inhibits the discharge pumps, which prevents specific proteins from passing through the membrane of intestinal epithelial cells, or enterocytes that release xenobiotics, mainly drugs [19]. Chemically, it is a linear copolymer composed of β-(1 → 4)-2-acetamido-d-glucose and β-(1 → 4)-2-amino-d-glucose units. It is known to have a variety of biological features, including biodegradability, biocompatibility, and a lower toxicity level [20]. CS is a safe, non-allergenic, biocompatible, and biodegradable polyominopolysaccharide. Because of these characteristics, CS is a useful pharmaceutical excipient and carrier for the creation of prodrugs. It also acts as an immune modulator, wound-healing accelerator, and antioxidant in addition to having anticancer, antibacterial, and anticholesterolemic effects [21]. The most biologically active component of vitamin E is a-tocopherol. It is a yellow, sticky substance that is insoluble in water but easily dissolves in solvents that are organic solvents. It has been beneficial in cancer prevention, anti-inflammatory properties, and antioxidant functions. Vitamin E has a number of roles, including direct impacts on enzyme activity, regulation of gene transcription, antioxidant, anti-inflammatory, anti-cancer, and anti-atherogenic effects. Vitamin E is essential for the central nervous system in addition to these other roles, and it has been proposed that vitamin E may help prevent and/or treat some neurological illnesses [22].

Antioxidants, such as vitamin E (α-tocopherol), have been demonstrated in experimental studies to reduce platelet aggregation and the propensity to produce thrombi and to enhance atherosclerotic plaque stability and vasomotor [26]. The most biologically active component of vitamin E is α-tocopherol. It has been beneficial in cancer-preventing, anti-inflammatory properties, and antioxidant functions [24]. In addition, CS was utilized as a matrix in nanoparticles made from polymers to encapsulate bioactive substances like α-tocopherol, enabling regulated delivery and protection of the latter [25]. Several scientific investigations have shown that α-tocopherol, a lipid nanoparticle, may be effectively incorporated into nanocarriers that provide effective protection against ultraviolet (UV)-radiation and antioxidant benefits to the skin. Alternatively, CS oleate nanoemulsion containing tocopherol potential effect on the wound healing system. In addition, to increase its distribution to cells and tissues, tocopherol has been added to phospholipid vesicles, where it functions as a membrane stabilizing agent [26]. The ratio of α-tocopherol to CS and the method of preparation employed in the formulation of the bio-nanocomposite may have an impact on its anti-inflammatory properties. Proper formulation conditions can improve the bioavailability and stability of both components, ensuring effective distribution to target tissues and cells [27]. Combining CS with α-tocopherol in a bio-composite may help to minimize inflammation. The antioxidant activity of α-tocopherol further reduces oxidative stress and lowers the formation of ROS, improving the anti-inflammatory effects of CS [28]. Combined, these two substances may have a greater anti-inflammatory impact than when administered separately. A bio-composite containing α-tocopherol and CS has the potential to have substantial anti-inflammatory properties. Their complementary mechanisms of action and synergistic interactions may result in the creation of innovative treatment strategies for inflammatory illnesses [29]. α-tocopherol-CS bio-composites have been the subject of recent studies in an effort to increase the therapeutic effectiveness of both substances. By utilizing the structural and functional benefits of CS, these bio-composites enhance the stability and bioavailability of α-tocopherol through continuous release. Both *in vitro* and *in vivo*, these systems have demonstrated promise in lowering inflammation, encouraging angiogenesis, and assisting tissue regeneration [30]. The incorporation of biocompatible materials into modern biomedical applications has gained traction as the desire for safer, multifunctional medicinal agents grows. When integrated into a bio-composite system, these compounds have synergistic uses in drug administration, wound healing, tissue engineering, and coagulation and inflammation pathway control [31].

This study aimed to assess the anti-inflammatory, antioxidant, anticoagulant, thrombolytic, cytotoxic, and embryonic toxicology potential of α-tocopherol-CS bio-composite. In addition, two active compounds were incorporated. The researchers aimed to investigate synergistic effects and expand the biomedical applications of the bio-composite.

METHODS

Materials and instruments

Sigma-Aldrich Chemical Reagent Co., Ltd. supplied us with easily available food-grade CS, acetic acid, α-tocopherol, and food-grade sodium tripolyphosphate (TPP). All of the reagents used in this study were of analytical purity. The following instruments were used in the present study such as orbital shaker (Lark), magnetic stirrer (Remi), double beam UV- visible spectrophotometer-2377, and COSLAB - Model: HL-10A light microscope.

Preparation of CS nanoparticles

500 mg of low molecular weight CS was weighed and dissolved. 1% of 1 mL acetic acid and 49 mL of deionized water were added. The composition is kept in a magnetic stirrer at 450 rpm for 2 h. Furthermore, Sodium TPP, used as a crosslinking agent, was dissolved in distilled water and added dropwise to the CS solution under continuous magnetic stirring. The nanoparticle electrostatic attraction between the positively charged amino groupings of CS and the negatively charged phosphate groups of TPP cross-linkage resulted in their unprompted formation. The solution is then kept in a magnetic stirrer for 2 h, synthesizing CS nanoparticles [32].

Preparation of α-tocopherol-CS bio-composite

Freshly prepared 100 mg of α-tocopherol was mixed with 5 mL of ethanol, then to prepare CS and α-tocopherol bio-composite, 2 mL of α-tocopherol was added to 8 mL of CS nanoparticles. After 12 h of being shaken and covered with aluminum foil, a color transformation was noticed in the beaker. This indicated the formation of α-tocopherol -based CS nanocomposite.

Anti-inflammatory activity

Bovine serum albumin (BSA) denaturation assay

The bovine serum albumin denaturation experiment was used to evaluate the anti-inflammatory properties of the generated α-tocophero- CS bio-composite. 0.05 mL of various ϑ-tocopherol CS bio-composite concentrations (10, 20, 30, 40, and 50 µg/mL) was combined with 0.45 mL of bovine serum albumin. The measured pH was 6.3. After standing at room temperature for 10 min, it was incubated for 30 min at 55°C in a water bath. Dimethyl sulfoxide served as the control group, and diclofenac sodium served as the standard group. After that, the samples were measured using spectrophotometry wavelength of around 660 nm [36]. Calculating the percentage inhibition of protein denaturation involved dividing the absorbance of the sample by the absorbance of the control, then multiplying the result by 100.

Egg albumin denaturation assay

Egg albumin denaturation test was performed using 2.8 mL of 1X phosphate buffer and 0.2 mL of fresh egg albumin. The α-tocopherol-CS biological composite was added to the reaction mixture in several quantities (10, 20, 30, 40, and 50 µg/mL). The final pH was 6.3. Ten minutes were spent at room temperature, and then it was incubated in a water bath at 55°C for 30 min. The standard group was diclofenac sodium, while the control group was dimethyl sulfoxide. The samples were then subjected to spectrophotometric measurements at 660 nm [33]. Calculating the percentage inhibition of protein denaturation involved dividing the absorbance of the sample by the absorbance of the control, then multiplying the result by 100.

Membrane stabilization assay

The *in vitro* stabilizing the membrane assay is a frequently used procedure to evaluate a substance's capacity to stabilize membranes, whether it be natural or manufactured. In this investigation, the ability of a chemical to preserve the stability of the cell barrier and prevent intracellular molecules from escaping through the disruption of the membrane is evaluated. Phosphate-Buffered Saline (PBS), Tris-HCl buffer (50 mM, pH 7.4), human red blood cells (RBCs), different doses of an α-tocopherol -CS bio-composite (10, 20, 30, 40, and 50 µg/mL), a centrifuge tube, and a UV-Vis spectrophotometer are among the items [34].

Preparation of RBC suspension

Freshly collected blood samples should be drawn and put in a sterilized container that contains an anticoagulant substance. The RBCs can be separated from other blood components by centrifuging the blood for 10 min at room temperature at 3000 RPM. Remove the supernatant from the RBCs and rinse them three times with PBS. Repeatedly suspending the RBCs in the Tris-HCl buffer will yield a 10% (v/v) RBC suspension.

Assay procedure

All centrifuge tubes should contain 1 mL of the RBC suspension, pipetted in. Then, each tube received varying doses of the *α*-tocopherol CS bio-composite (10, 20, 30, 40, and 50 µg/mL). After gently mixing, let the tubes sit at 37°C for 30 min. To pellet the RBCs, centrifuge the tubes for 5 min at room temperature, set at 2500 rpm. A UV-Vis spectrophotometer may be used to measure the supernatant's absorbance at 560 nm.

The absorbance of the sample was subtracted from the absorbance of the control, the result was divided by the absorbance of the control, and the result was then multiplied by 100 to calculate the percentage inhibition of hemolysis. The absorbance of the red blood cell suspension without any added test chemicals is used as the control. The sample optical density indicates the absorbance of the RBC suspension containing the test substance.

Anticoagulation

Freshly collected 2 mL of venous blood was used to test the α-tocopherol-CS bio-nanocomposite anticoagulant properties at room temperature. In this experiment, 2 mL of fresh venous blood was transferred directly into a clean, dry 15mL centrifuge tube. The tube marked Tube A is a control, and Tube B is an α-tocopherol-CS bio-nanocomposite. After that, 1 mL of α-tocopherol-CS bio-nanocomposite solution was added to tube B was mixed thoroughly. After that, the two blood samples were kept at room temperature for an hour to look for any obvious changes [35].

Thrombolytic activity

1 drop of blood was taken and placed onto a sterile glass slide. It was then incubated at room temperature for 45 min. The blood was clotted, and then 20 µg/mL of α-tocopherol-CS bio-composite solution was added. Then, without any further solution, compared with the control. The glass slide was incubated for a period of 90 min at room temperature; the duration of the incubating was noted to monitor the clot's lysis [36].

Anti-oxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

From a 0.1 mM stock, a new DPPH working solution (20 µM in methanol) was made. A 96-well plate was filled with 200 µL of this working solution and different concentrations of the α-tocopherol-CS bio-composite (10, 20, 30, 40, and 50 µg/mL). The combinations were allowed to sit at room temperature in a poorly light area for 10 min. The percentage of DPPH radical scavenging activity was then determined using the following formula after absorbance was measured at 517 nm with methanol serving as the blank:

Percentage of DPPH Scavenging Activity = ([Absorbance of control–Absorbance of sample]/Absorbance of control) × 100

Where control is DPPH solution without the sample, and the sample is DPPH solution with the α-tocopherol-CS bio-composite. Ascorbic acid of the same different concentrations was used as a standard.

Hydrogen peroxide radical scavenging assay

The biosynthesized α-tocopherol-CS nanobiocomposite was assessed for its capacity to scavenge hydrogen peroxide (H_2O_2). A phosphate buffer with a pH of 7.4 was mixed with 40 mM H_2O_2. A mixture of the test sample (α-tocopherol-CS bio-composite) and a standard sample of ascorbic acid at varying amounts (10µg/mL, 20µg/mL, 30µg/mL, 40µg/mL, and 50 µg/mL) was combined with 0.6 mL of H_2O_2 solution. The absorbance was measured at 230 nm using spectrophotometrically after incubating for 10 min in a dark atmosphere with Vitamin C as a standard [37].

Ferric reducing antioxidant power (FRAP) assay

Reagent for assay

300 mM of Acetate buffer with pH 3.6: To 3.1 g sodium acetate trihydrate, 16 mL of glacial acetic acid was added to make the volume 1 L with dis. H_2O.TPTZ: (molecular weight [Mol.Wt.] 312.34), 10 mM in 40 mM HCl (Mol.Wt. 36.46). Ferric Chloride. 6 H_2O: (Mol.Wt. 270.30), 20 mM.Immediately before use, the FRAP working reagent was freshly prepared by combining the acetate buffer, TPTZ solution, and ferric chloride solution in a 10:1:1 (v/v/v) ratio. Ferrous sulfate heptahydrate ($FeSO_4 \bullet 7H_2O$), prepared in methanol at concentrations ranging from 0.1 to 1.5 mM, served as the standard.

Procedure

0.7 mL of the α-tocopherol-CS nanobio-composite was combined with

2.3 mL of the FRAP reagent at several dosages (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL) and incubated at 37°C for 30 min in the dark. Using a spectrophotometer, the absorbance was recorded at 593 nm against a standard that included all of the chemicals but not the sample. A higher reaction mixture absorbance suggests a higher capacity for reduction. Triples of the samples were measured. The reference was ascorbic acid [38].

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid (ABTS) assay

When 7.0 mM ABTS (50% ethanol) and 2.45 mM potassium persulfate (in distilled water) were combined, the ABTS radical cation (ABTS+) was formed. This reagent was kept in the refrigerator for a minimum of 24 h. This reagent is diluted using 50% ethanol until an absorbance of 1.0 (±0.02) is achieved at 734 nm. Twenty µL of the sample α-tocopherol-CS bio-composite and 250 µL of ABTS+ were applied to 96-well microplates. The sample solution was diluted in distilled water, and the concentrations were 10, 20, 30, 40, and 50 µg/mL. The standard was ascorbic acid. Ethanol (20µL) served as the blank. Using a microplate reader, the reading was taken at 734 nm following a 10-minute reaction in the dark. The radical scavenging activity was calculated as follows:

$$I (\%) = ([Abs0 - Abs1]/Abs0) \times 100$$

Where Abs0-absorbance of the blank; Abs1-absorbance in the presence of the test compound at different concentrations.

Nitric oxide (NO) radical inhibition assay

The Griess-Ilosvay reaction can be used to evaluate the suppression of NO radicals. Instead of utilizing 1-naphthylamine (5%), naphthyl ethylene diamine dihydrochloride (0.1%w/v) was used in this experiment to modify the Griess-Ilosvay reagent. For 1½ h, the reaction mixture (3 mL) that contained ascorbic acid (0.5 mL) as the standard solution, phosphate buffer (0.5 mL), sodium nitroprusside (10 mm, 2 mL), and α-tocopherol- CS bio composite (10, 20, 30, 40, and 50 µg/mL) was incubated at 25°C. In diffused light, 0.5 mL of the pink chromophore forms after incubation. At 540 nm, the absorbance of these samples was measured with appropriate blank solutions.

The mixture was mixed with 1 mL of sulfanilic acid (0.33 % in 20 % GAA) and was kept for five min for diazotization to complete. Then, 1 mL of naphthyl ethylenediamine dihydrochloride was added and kept at 25°C for 30 min.

ZEBRAFISH EMBRYONIC TOXICOLOGY EVALUATION OF α-TOCOPHEROL-CS BIOCOMPOSITE

Fish maintenance and α-tocopherol-CS nanocomposite exposure

Wild-type zebrafish (Danio rerio) were purchased from nearby Indian suppliers and kept in separate tanks with regulated pH (6.8–8.5), light/dark cycle (14:10 h), and temperature (280±20C°C). The fish were fed optimal food or commercially available dry bloodworms twice a day. One female and three males were crossed in each breeding tank to create zebrafish embryos. Viable eggs were then retrieved and rinsed at least three times with freshly made E3 medium devoid of methylene blue. Fertilized eggs containing 20 embryos/2 mL solution per well were placed in culture plates with different well sizes (6, 12, and 24 wells) for the investigation. Three replications of the experimental and control groups were made.

The study intervention was prepared by adding a newly prepared stock suspension of α-tocopherol-CS bio-composite in five different concentrations straight to the E3 medium. The nanoparticles were dispersed by sonicating the solution for 15 min while keeping the pH between 7.2 and 7.3. Various quantities of α-tocopherol-CS bio-composite, ranging from 5, 10, 20, 40, and 80 µg/mL, were administered to healthy fertilized embryos for 24 to 96 h after fertilization. When the embryos were incubated in the E3 medium, the α-tocopherol -CS bio-composite was introduced. In addition, the experiment included control groups. Every 12 h, the groups exposed to the nanoparticles had their dead embryos removed. Every trial plate was kept at 28°C and covered with foil to keep out light.

Zebrafish embryo evaluation

Using a stereo microscope, Zebrafish embryos have been investigated at various stages of development, throughout the exposure time that followed fertilization. For 24–78 h, the embryos were exposed to different doses of α-tocopherol -CS bio-composite (5, 10, 20, 40, and 80 µg/mL). The rates of hatching and embryonic death were measured every 24 h. The study's outcomes included the mortality of the embryos and hatchlings, the rate of hatching, and the detection and recording of any abnormalities in the larvae and embryos in the treatment and control groups. A COSLAB - Model: HL-10A light microscope was used to take pictures of embryos with malformations, and every 24 h, the proportion of aberrant embryos was noted [38].

Cytotoxic effect

Brine shrimp lethality assay

Saltwater preparation

Two grams of iodine-free salt and 200 mL of purified water were weighed. Six-well Enzyme-Linked Immunosorbent Assay (ELISA) plates were filled with 10–12 mL of saline water. Ten nauplii (5, 10, 20, 40, and 80 µg/mL) were progressively introduced to each well. The necessary concentration was subsequently followed by the addition of the α-tocopherol -CS bio-composite. The plates were incubated for a whole day. Following a 24 h observation of the ELISA plates, the number of live nauplii was measured and computed using the formula below [39],

The ratio of the number of dead nauplii to the number of live nauplii is 100.

Statistical analysis

Statistical analysis was executed, two-way analysis of variance ANOVA using Tukey's post hoc test for all groups compared, with IC_{50} value calculated by using non-linear regression for statistical analysis test run in replicate using GraphPad Prism version 8.0 software. It was determined that a P value of 0.05 was statistically significant.

RESULTS AND DISCUSSION

Visual observation

Visual observation serves as a preliminary tool for analyzing bio-composite synthesis. The precursor α-tocopherol-CS solution initially appeared pale yellow, gradually changing to cloudy white at the final stage. This color transformation indicates successful reduction and stabilization during bio-composite formation. The observed changes confirm the synthesis of the α-tocopherol-CS bio-composite (Fig. 1).

UV-spectral analysis for further confirmation of the α-tocopherol-CS bio-composite. The analytical method enables a more thorough evaluation of the optical characterization of the bio-composite. Blue color peaks in Fig. 2 show the UV analysis of CS, which revealed the strong two absorption peaks at a wavelength range of 354 and 439 nm [40]. Similarly, block peaks are observed in a wavelength range of 362–450 nm, providing primary confirmation of the natural substance of the α-tocopherol-CS biocomposite.

Anti-inflammatory activity

The comparison of alpha tocopherol and alpha tocopherol-chitosan bio-composite was evaluated moderately, Fig. 3 exhibiting protein denaturation at a high concentration of 50 µL of alpha tocopherol with 77.7 ± 0.35 (in the bovine serum albumin assay), Fig. 4 exhibits 75.45 ± 0.77 (in the egg albumin assay), and Fig. 5 exhibits 82.45 ± 0.77 (in the membrane stabilization assay). However, the Lowest concentration of 10 µg/mL, with 40.6 ± 0.56 of inhibition (in the bovine serum albumin assay), 49.3 ± 0.98 of inhibition (in the egg albumin assay), and 51.95 ± 1.48 of inhibition (in the membrane stabilization assay) exhibiting inhibition bio-composite exhibited slightly lower protein denaturation compared to the standard diclofenac sodium. The alpha

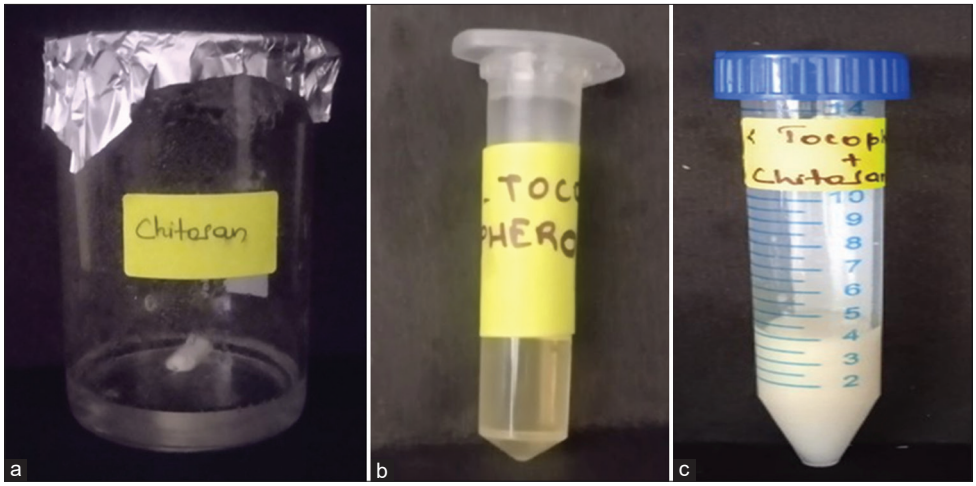

Fig. 1: Visual observation images of alpha-tocopherol chitosan (α-tocopherol-CS) bio-composite (a) CS, (b) α-tocopherol, (c) α-tocopherol-CS bio-composite ultraviolet-spectral analysis of α-tocopherol-CS bio-composite

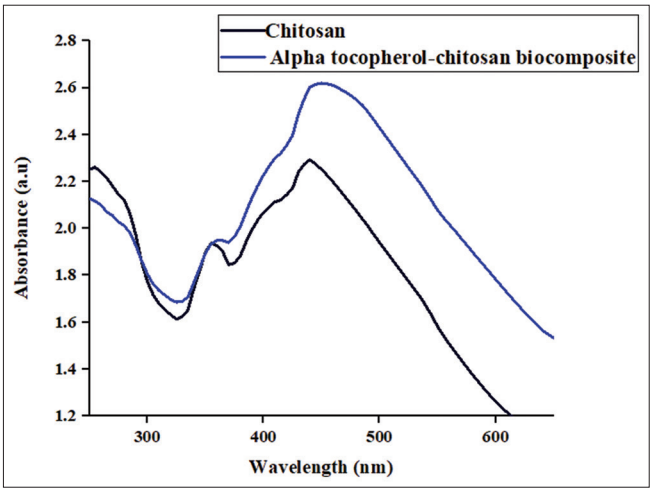

Fig. 2: Ultraviolet-visible spectral analysis of alpha-tocopherol-chitosan bio-composite

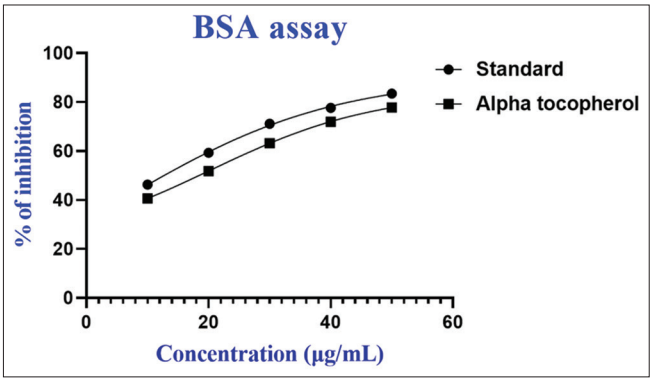

Fig. 3: Graphical presentation of alpha-tocopherol, anti-inflammatory activity by using bovine serum albumin assay, two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects, p-value**<0.0001 column factor **(p<0.0079, and interaction between column and row factor was not significant (p=0.05780)**

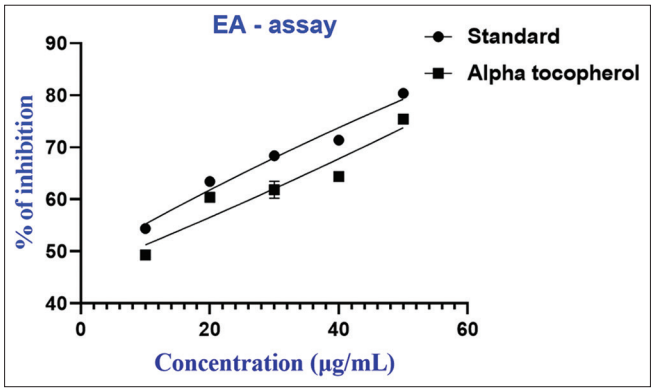

Fig. 4: Graphical representation of alpha-tocopherol, anti-inflammatory activity determined by using egg albumin assay, two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects, p-value**<0.0001 column factor *(p<0.0155), and interaction between column and row factor was significant **(p=0.0016)**

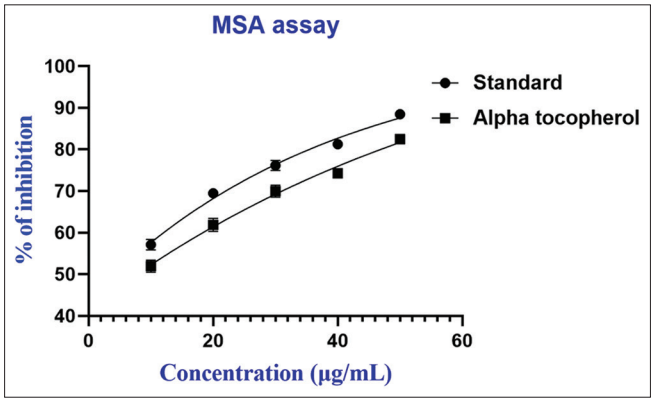

Fig. 5: Graphical presentation of alpha-tocopherol, anti-inflammatory properties determined by membrane stabilization assay, two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects, p-value**<0.0001 column factor *(p≤0.0170), and interaction between column and row factor was significant *(p=0.0110)**

tocopherol-chitosan bio-composite anti-inflammatory properties were assessed through the use of three different kinds of tests: The albumin denaturation test in bovine serum albumin Fig. 6, the egg albumin denaturation assay Fig. 7, and the membrane stabilization assay Fig. 8, the experiments demonstrated the least protein denaturation at a

high concentration of 50 µg/mL, with 74.2 ± 1.02 of inhibition and IC50 value of ~ 1.292e-160, (in the bovine serum albumin assay), 76 ± 1.11 of inhibition and IC50 value of ~ 0.000, (in the egg albumin assay), and 84.13± 1.00of highest inhibition and IC50 value of ~ 1.216e-226, (in the membrane stabilization assay) exhibiting inhibition is non-linear regression statistical analysis. However, the Lowest concentration of 10 µg/mL, with 50.2 ± 1.01 of inhibition (in the bovine serum albumin assay), 41.13 ± 1.10 of inhibition (in the egg albumin assay), and 52.16± 0.86 of inhibition (in the membrane stabilization assay) exhibiting inhibition bio-composite exhibited slightly lower protein denaturation compared to the standard diclofenac sodium. As demonstrated by these tests, anti-inflammatory efficacy has a dependent on dose effect, and at all tested dosages, the percentage of denaturation of proteins reduction stays quite close to that of diclofenac sodium. The mechanistic action of inflammation is closely associated with protein denaturation and the formation of autoantigens. The α-tocopherol–chitosan biocomposite prevents thermal and chemical denaturation of albumin by stabilizing the protein's native conformation through hydrogen bonding and electrostatic interactions between chitosan's amino groups and the protein backbone [3]. The α-tocopherol component, a potent lipophilic antioxidant, donates hydrogen atoms to neutralise reactive intermediates that cause unfolding and aggregation. Together, these synergistic effects preserve the structural integrity of albumin, similar to the protective mechanism of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit protein denaturation and suppress prostaglandin synthesis by inhibiting COX and LOX enzymes [48].

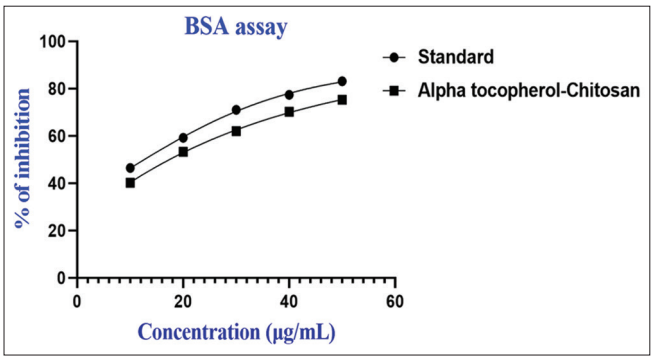

Fig. 6: Graphical presentation of anti-inflammatory properties of alpha-tocopherol-chitosan bio-composite using bovine serum albumin assay, two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects, p-value0.0051 column factor **(p≤0.0029, and interaction between column and row factor was not significant (p=0.0729)**

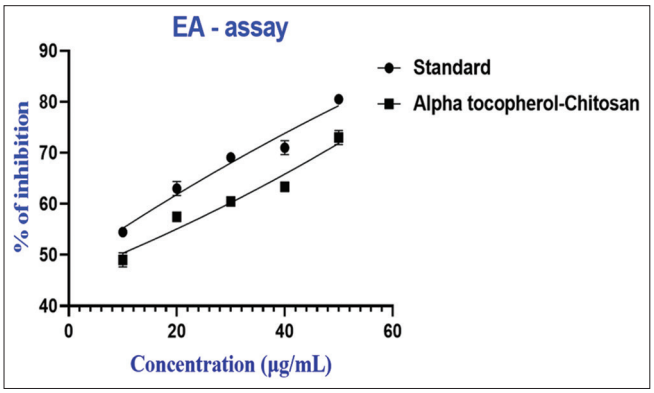

Fig. 7: Graphical presentation of anti-inflammatory activity of alpha-tocopherol-chitosan bio-composite using egg albumin denaturation assay, two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects of time, p-value**<0.0001 column factor *(p≤0.0145), and interaction between column and row factor was also significant *(p=0.0405)**

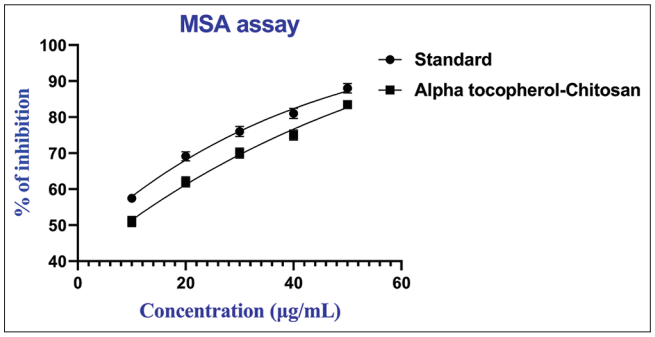

Fig. 8: Graphical presentation of the anti-inflammatory properties of alpha-tocopherol-chitosan bio-composite using a membrane stabilization assay, two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects, p-value**<0.0001 column factor **(p≤0.0029), and interaction column and row factor significant*(p=0.0344)**

In this experiment, Fig. 9a, 2.0 mL of fresh venous blood was transferred centrifuge tube. It must be maintained for 6–15 min at room temperature. Then, after 15 min, both tubes must be inverted. If a solid is observed in the control tube A, the test indicates normal coagulation, and tube B is also inverted. The result was observed in tube B. If the clot breaks down, if there is no coagulation, it indicates α-tocopherol-CS bio-nanocomposite has effective anticoagulant activity. In Fig. 9b, the thrombolytic activity of α-tocopherol with CS bio-nanocomposite in five variable doses (10, 20, 30, 40, and 50 µg/mL) suggests its potential as an effective clot-dissolving agent. ROS can directly damage fibrin, causing the clot to break down. Furthermore, α-tocopherol-CS bio-nanocomposite has been shown to activate the plasminogen-plasmin system, which promotes clot disintegration. The activation stimulates the conversion of plasminogen to plasmin, which further cleaves fibrin and aids in clot breakup for 1–5 min [45].

Fig. 10 shows the graphical presentation of the thrombolytic activity of α-tocopherol-CS bio-composite. A clot lysis assay was done to assess at five different concentrations from 10 µg/mL to 50 µg/mL with the clot lysis values of 20, 16, 13, 10, and 6 mins, respectively. The clot lysis was found to be the highest at 50 µl/mL, so increasing the concentration exhibits at 6 min of thrombolytic activity [45].

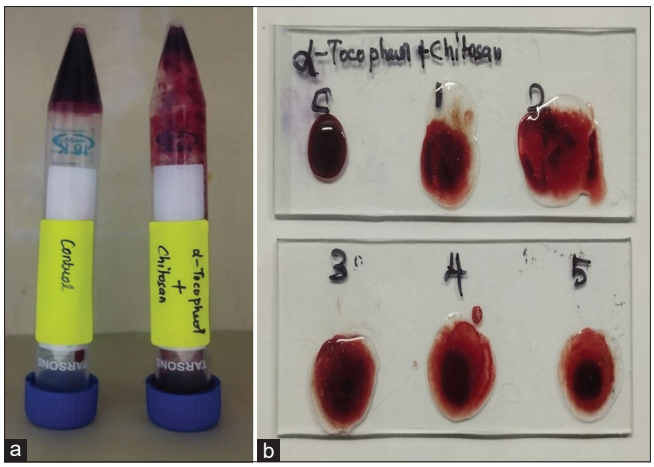

Fig. 9: Schematic representation of chitosan (CS) - α-Tocopherol-CS bio-composite. (a) Anticoagulant activity, (b) Thrombolytic activity

Antioxidant activity assay

In Fig. 11, the antioxidant activity of α-tocopherol-based CS bio-composite was assessed using the DPPH assay. The α-tocopherol–chitosan biocomposite neutralizes DPPH• radicals through hydrogen atom and electron transfer. α-Tocopherol donates hydrogen atoms from its hydroxyl group on the chromanol ring, while chitosan's amino and hydroxyl groups contribute additional electrons, collectively converting DPPH• to its reduced, colourless form. This indicates strong free radical–quenching ability. Ascorbic acid was utilised as a standard. The bio-composite nanoparticles by DPPH demonstrated the highest inhibition at 50 µg/mL (90.68 ± 1.32) and the lowest inhibition at 10 µg/mL (62.27 ± 1.03) in the DPPH assay. IC50 value of ~ 5.154e-117was analysed by using non-linear regression. Two-way ANOVA using Tukey's *post hoc* test for all groups revealed highly significant effects ****(p-value < 0.0001).

In Fig. 12, H_2O_2 assay assessed the antioxidant activity of α-tocopherol-based CS bio-composite. The α-tocopherol–chitosan biocomposite scavenges hydrogen peroxide by donating electrons and hydrogen atoms from α-tocopherol and chitosan's hydroxyl and amino groups, converting H_2O_2 into harmless water molecules. This prevents the formation of highly reactive hydroxyl radicals and protects biomolecules from oxidative damage. The standard used was vitamin C ascorbic acid, and the H_2O_2 assay-mediated at α-tocopherol-based CS bio-composite exhibits the highest percentage of inhibition, with 50 µg/mL, whichis85.33±1.34 and the lowest inhibition at 10 µg/mL, 45.53±0.72 H_2O_2 assay.IC_{50} value of 9.024e+034 was analyzed by using non-linear regression. Furthermore, Two-way ANOVA using Tukey's *post hoc* test for all groups revealed highly significant effects ****(p<0.0001).

In Fig. 13, the FRAP assay is measured by the Ferric reducing potential of α-tocopherol-based CS bio-nanocomposite. The standard solution of ascorbic acid was used for the FRAP assay at α-tocopherol-mediated CS bio-nanocomposite. The α-tocopherol–chitosan biocomposite reduces Fe^{3+} to Fe^{2+} through electron donation from α-tocopherol and the hydroxyl and amino groups of chitosan, demonstrating strong reducing potential and overall antioxidant power. Results exhibited the lowest percentage of inhibition (10 µg/mL 68.18 ± 1.66 and parallelly are the highest scavenging activity of 86.09 ± 1.39 was found to be at 50 µg/mL. The greatest antioxidant activity was found in the

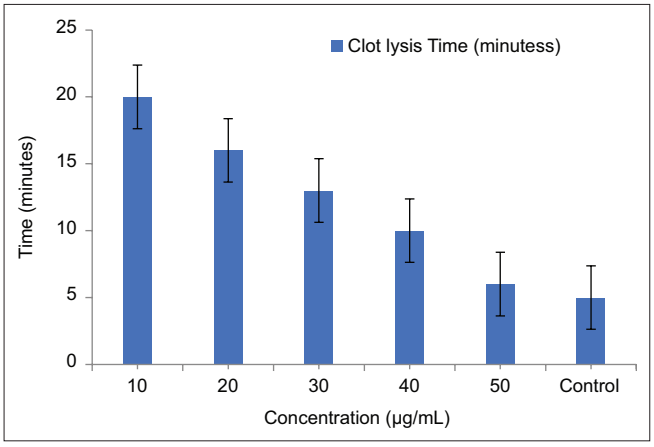

Fig. 10: Graphical representation of thrombolytic activity using alpha-tocopherol-chitosan bio-composite

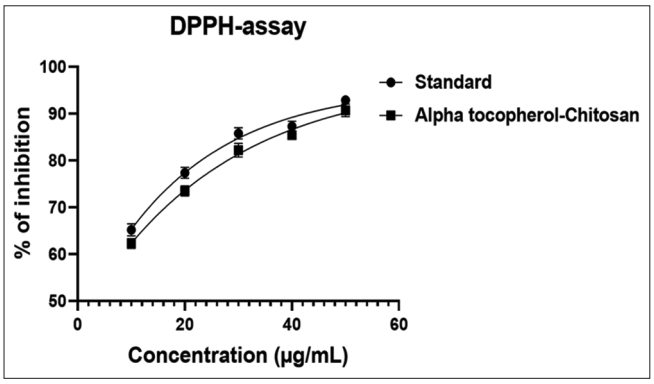

Fig. 11: Antioxidant activity of 2,2-diphenyl-1-picrylhydrazyl assay alpha-tocopherol-chitosan bio-composite demonstrating the percentage of inhibition at various concentrations, with equivalent antioxidant activity, in which the bio-composite a standard is compared

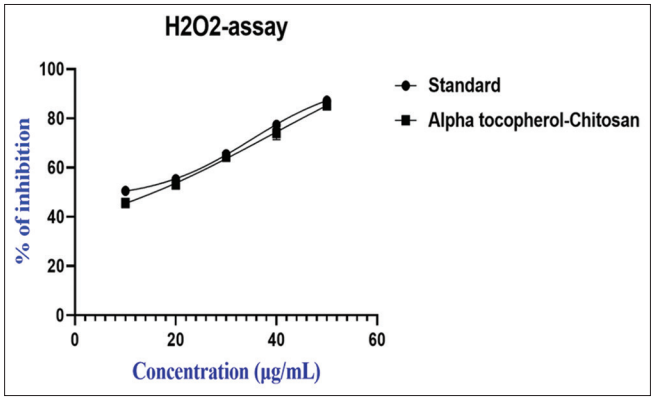

Fig. 12: Graphical representation of the antioxidant activity of alpha-tocopherol-based chitosan bio-composite using hydrogen peroxide assay

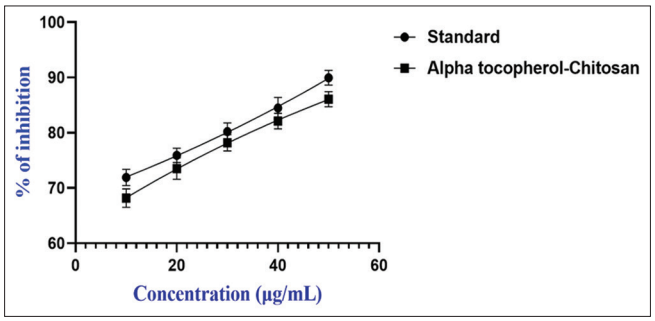

Fig. 13: Graphical representation of the antioxidant activity of alpha-tocopherol-based chitosan bio-nanocomposite using ferric-reducing antioxidant powder assay

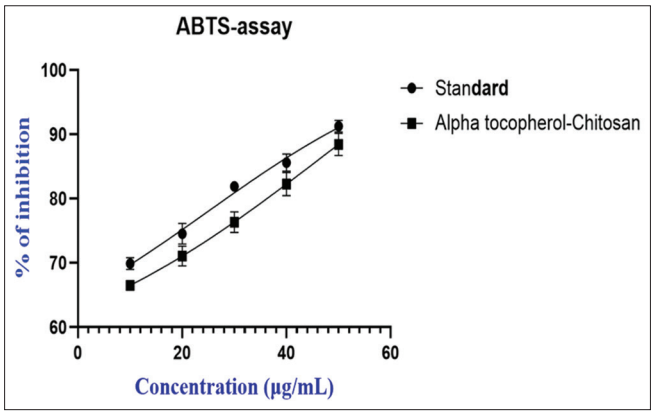

Fig. 14: Graphical representation of the antioxidant activity of alpha-tocopherol-based chitosan bio-composite by using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid assay

α-tocopherol-mediated CS bio-nanocomposite of the FRAP antioxidant activity assay. Two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects of time, ****(p-value<0.0001, column factor **(p = 0.008), and interaction between column and row factor is highly significant *(p = 0.0314). IC_{50} value of ~ 6.029e + 305 was analysed by using non-linear regression.

Fig. 14 shows the graphical representation of the ABTS assay employed to assess the antioxidant properties of bio-composite α-tocopherol-based CS. In the ABTS•$^{+}$ assay, the α-tocopherol–chitosan biocomposite reduces the blue-green ABTS•$^{+}$ radical cation to its neutral form via electron and hydrogen transfer. The synergistic action of α-tocopherol's lipid-soluble antioxidant capacity and chitosan's polar functional groups enhances both hydrophilic and lipophilic radical scavenging efficiency, demonstrating broad-spectrum antioxidant potential. The standard was ascorbic acid; the α-tocopherol-based chitosan bio-composite exhibited the highest inhibition of 88.45 ± 1.72 and was found to be 50 µg /mL. Results exhibited the lowest percentage of inhibition, 10 µg/mL, 66.51 ± 0.75. Two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects of ****(p-value < 0.0001, column factor *(p = 0.0467), and interaction between column and row factor is significant *(p = 0.0263). IC_{50} value of ~ 6.029e+305 was analysed by using non-linear regression.

In Fig. 15, in evaluating the antioxidant activity of nitric acid scavenging activity, using naphthyl ethylene diamine dihydrochloride (0.1%w/v) in place of 1-naphthylamine (5%), the Griess-Ilosvay reagent was altered. The α-tocopherol–chitosan biocomposite scavenges nitric oxide radicals by donating electrons and hydrogen atoms from α-tocopherol and chitosan functional groups, converting reactive NO into stable nitrite forms. This prevents the formation of peroxy nitrite and thereby reduces nitrosative stress and cellular oxidative damage. The

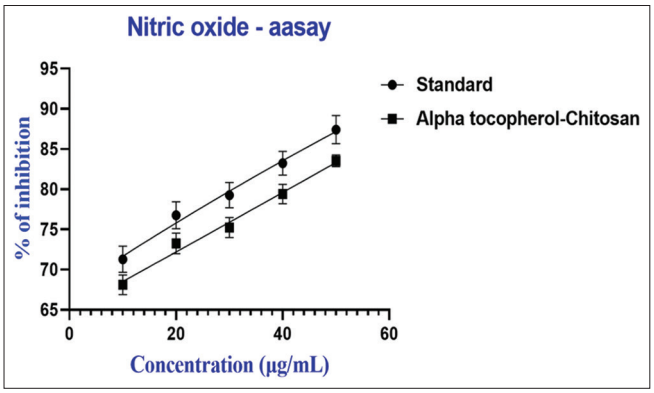

Fig. 15: Graphical representation of the antioxidant activity of alpha-tocopherol-based chitosan bio-nanocomposite using NO scavenging activity assay

α-Tocopherol-chitosan bio-composite obtained maximum and highest inhibition at 50 µg/mL, is an 83.53 ± 0.73, and the lowest concentration obtained moderate inhibition at 68.10 ± 1.23 IC50 value of ~ 2.154e + 139 was analysed by using non-linear regression. Two-way measures ANOVA using Tukey's *post hoc* test for all groups compared, revealed highly significant effects of ****(p-value<0.0001). This study compared other antioxidant assays, α-tocopherol-CS bio-composite, using nitric acid scavenging activity, which exhibits the highest free radical scavenging activity. DPPH assay, H_2O_2 assay, FRAP assay, ABTS assay, and NO assays were done to analyze the antioxidant properties, which showed highly antioxidant properties of the synthesized bio-composite at increasing concentrations from 10 µL to 50 µL, but the values were not more than the standard values. The significantly increasing antioxidant activity was evident as the concentration increased.

In Fig. 17, the α-tocopherol-CS bio-composite, cytotoxic effects were investigated using the brine shrimp lethality method, a commonly used technique to gauge a substance's cytotoxicity by measuring its effect on brine shrimp nauplii survival. To create a baseline for determining the percentage of live nauplii, a control group that received no therapy was kept in place for this investigation. The results of the cytotoxicity assessment indicated that different concentrations of the bio-composite exhibited varying effects on nauplii survival. Sixty live nauplii were collected and evenly distributed, with 10 nauplii per well. The impact of the α-tocopherol-CS bio-composite was monitored at 24 h intervals. All 60 nauplii survived on the 1st day at all concentrations (5, 10, 20, 40, and 80 µL) [42]. Similarly, 100% of the nauplii survive on the 2nd day when the α-tocopherol-CS bio-composite is at a dose of 5 µg/mL. Similarly, the bio-nanocomposite retained approximately 100% of the live nauplii at doses of 20 µg/mL and 40 µg/mL. However, only 90% of the nauplii survived at a greater dose of 80 µg/mL. The impact of the α-tocopherol-CS bio-composite was monitored at 48h intervals.

In zebrafish embryos at 80 µg/mL of α-tocopherol-based CS bio-composite, zebrafish embryo viability was 80%, while at 40 µg/mL, viability also improved to 80%. Complete (100%) vitality was found at 5, 10, and 20 µg/mL, as well as in the blank control group. Fig. 17 shows the effects observed in the test and control groups, comparing those that received no treatment with those that received treatment. When exposed to doses of 5, 10, and 20 µg/mL, there were no discernible (p>0.05) alterations. But when exposed to increasing the bio-composite concentration to 40 and 80 µg/mL, the embryo death rate was significantly affected (p<0.01). The morphological imaging showed that on the 1st day, there were viable embryos inside the egg that were only partially formed, on the 2nd day, there were fully developed viable embryos inside the egg, and on the 3rd day, healthy zebrafish emerged. There were no visible signs of edema or somatic abnormalities such as a bent spine or tail (Fig. 16).

At an 80 µg/mL concentration, 60% of embryos successfully hatched at the concentration of α-tocopherol-CS bio-composite, 80% at 20 and

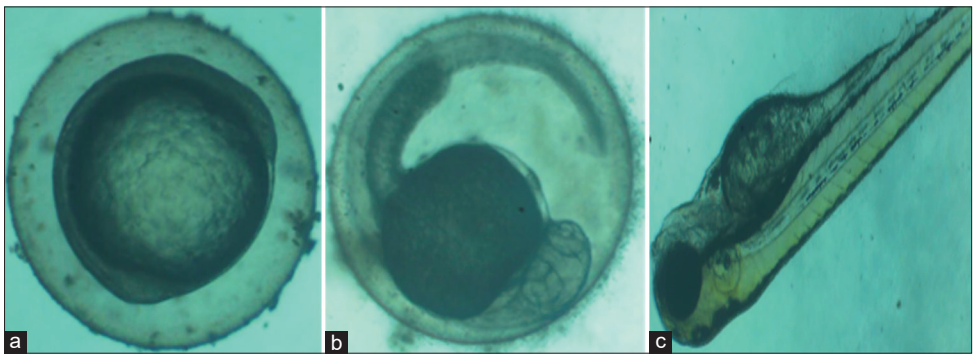

Fig. 16: Microscopic images of zebrafish embryonic toxicological imaging of the *α*-tocopherol-chitosan bio-nanocomposite. (a) On day 1, healthy embryos in zebrafish can be seen inside the eggs; (b) On day 2, the embryos are well developed while still inside the eggs; and (c) On day 3, the zebrafish have fully hatched and appear healthy, with no signs of malformations

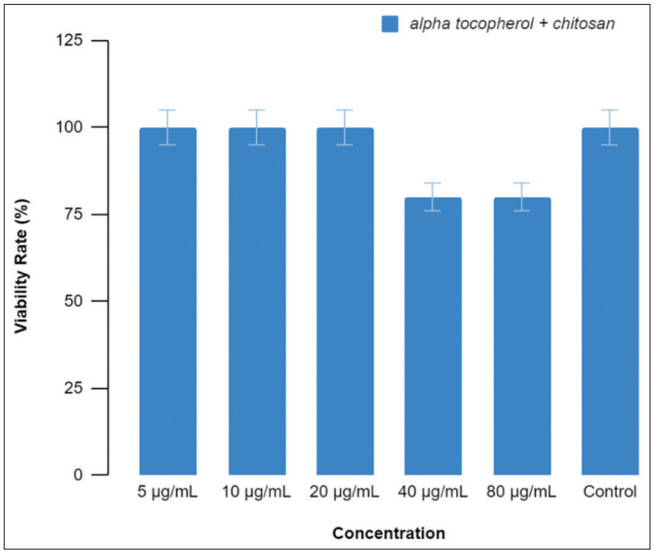

Fig. 17: Graph illustrating 76 h zebrafish embryo survival rates following exposure to varying doses of *α*-tocopherol-based chitosan bio-composite

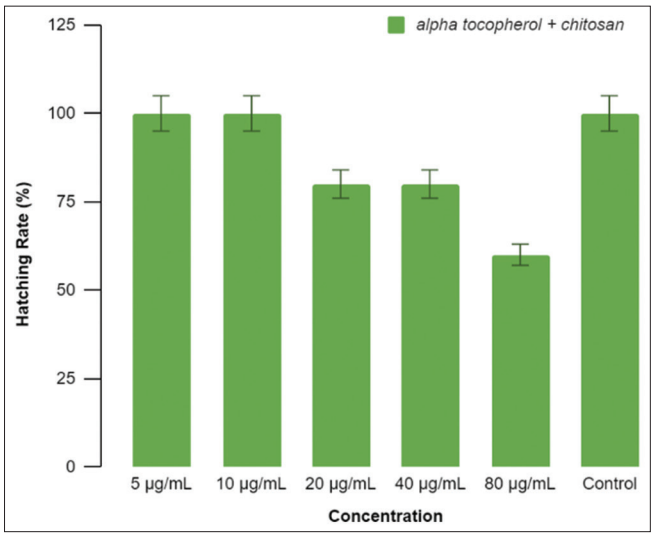

Fig. 18: Graph illustrating the hatching rates of zebrafish embryos exposed to different doses for 76 h, of *α*-tocopherol-based chitosan bio-composite

40 µg/mL, and 100% at 5 and 10 µg/mL concentrations. 100% of the hatching rate at the concentration of α-tocopherol-CS bio-composite, 5 µg/mL; similarly, by comparison, the hatching rate of the blank control was 100%. Likewise, Fig. 18 shows that the concentrations had an impact on the pace at which zebrafish embryos hatched. The untreated embryos had a 100% hatching rate. Nevertheless, embryos exhibited an 80% hatching rate at 20 and 40 µg/mL of the extract concentration. They had a p-value below 0.01. However, <0.05 indicated that the hatched percentage was reasonably significant up to a dose of 80 µg/mL [41].

In Fig. 19, the alpha tocopherol-chitosan bio-composite cytotoxic effects were investigated using the brine shrimp lethality method, a commonly used technique to gauge a substance's cytotoxicity by measuring its effect on brine shrimp nauplii survival. To create a baseline for determining the percentage of live nauplii, a control group that received no therapy was kept in place for this investigation. The results of the cytotoxicity assessment indicated that different concentrations of the

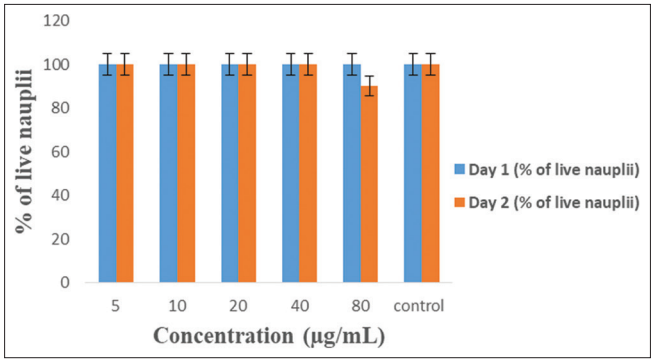

Fig. 19: Graph illustrating the cytotoxic effect of alpha-tocopherol-based chitosan bio-composite using brine shrimp lethality assay

bio-composite exhibited varying effects on nauplii survival. Sixty live nauplii were collected and evenly distributed, with 10 nauplii per well. The impact of the alpha-tocopherol-chitosan bio-composite was monitored at 24-hour intervals. All 60 nauplii survived on the first day at all concentrations (5, 10, 20, 40, and 80 µL) [48]. Similarly, 100% of the nauplii survive on the second day when the alpha-tocopherol-chitosan bio-composite is at a dose of 5 µg/mL. Similarly, the bio-nanocomposite retained approximately 100% of the live nauplii at doses of 20 µg/mL and 40 µg/mL. However, only 90% of the nauplii survived at a greater dose of 80 µg/mL. The impact of the alpha-tocopherol-chitosan bio-composite was monitored at 48 h intervals.

DISCUSSION

The potential of nanoparticles as anti-inflammatory drugs has drawn a lot of attention in recent years. The discussion focuses on the bio-composite from α-tocopherol-CS, with specific prominence on its anti-inflammatory properties. The information described here is aimed at explaining the α-tocopherol-CS bio-composite's potential therapeutic benefit in reducing inflammation as well as its applicability for a range of biomedical uses. The bio-composite showed a significant anti-inflammatory effect in the BSA denaturation assay, suggesting that it can prevent protein denaturation. Interestingly, at lower doses (10–50 µg/mL), the α-tocopherol-CS bio-composite showed a greater degree of inhibition, indicating a possible benefit in reducing inflammation by preventing protein denaturation.

Previously reported, developed α-tocopherol-CS-loaded soybean oil monostearin oleogel was designed to investigate the protective impact of different concentrations. To establish the standard curve for α-tocopherol, UV spectrophotometric measurements were carried out on various concentrations of α-tocopherol (0.2, 0.5, 0.8, 1.0, 1.5, 1.8, 2.0, and 2.5 mg/mL) using ethanol as a blank. The excitation wavelength range was 200–450 nm [41]. In the current study, preliminary physicochemical characterization confirmed the formation of a CS and α-tocopherol-CS bio-composite. Peaks were observed for CS at 354 and 439 nm, and the α-tocopherol-CS bio-composite peaks were confirmed at 362 and 450 nm.

In addition, the bio-composite continuously demonstrated superior suppression. This approach uses egg albumin as a test for protein denaturation, especially at the previously mentioned lower concentrations, confirming its anti-inflammatory capabilities [42]. According to these results, the α-tocopherol-CS bio-composite may be an asset for reducing inflammatory reactions. The bio-composite successfully stabilized red blood cell membranes in the membrane stabilizing assay, demonstrating its capacity to preserve cellular integrity in stressful situations. The membrane-stabilizing activity protects red blood cells from hypotonicity-induced hemolysis. The chitosan matrix interacts electrostatically with negatively charged phospholipids on the erythrocyte membrane, enhancing structural

integrity and reducing osmotic fragility. Concurrently, α-tocopherol, a potent lipid-soluble antioxidant, scavenges membrane-associated free radicals and prevents lipid peroxidation. This dual action preserves the stability of the erythrocyte membrane, resembling the lysosomal membrane stabilization achieved by conventional anti-inflammatory drugs, thereby minimizing the release of proteolytic enzymes and subsequent tissue injury [49].

The outcomes demonstrate the bio-composite's potential as a dependable anti-inflammatory drug, especially by stabilizing membranes and preventing protein denaturation pathways. Two-way measures ANOVA revealed highly significant effects of time, $p<0.0001$. Subject variability was not significant ($p=0.216$).

In this study, α-tocopherol-CS bio-composite antioxidant capability evaluated by DPPH, H_2O_2, FRAP, ABTS, and NO assays its capacity to scavenge. It could protect periodontal tissues from oxidative injury by reducing oxidative stress and free radicals. Although periodontal research on this bio-composite is still in its early stages, preliminary findings and experimental evidence have indicated encouraging treatment effects [43]. There were used, two-way ANOVA revealed replicative for highly significant differences for all concentrations. Their p-value was ***<0.0001. In the present study, thrombolytic investigation was determined, and a clot lysis assay was performed between 10 μg/mL and 50 μg/mL, representing five distinct concentrations, with clot lysis times of 20, 16, 13, 10, and 6 min. The maximum clot lysis was seen at 50 μg/mL, indicating that increasing the concentration leads to thrombolytic activity after 6 min.

Previously reported, tocopherol acetate-loaded transfers were synthesized with different adolescents (20, 40, 60, 80), resulting in stable, unilamellar vesicles (~85 nm) with high entrapment efficiency (72–90%). These nanocarriers demonstrated superior biocompatibility, antioxidant activity, and improved skin transport. They also increased cell proliferation and migration, which accelerated wound healing [26].

Already investigated, α-tocopherol-loaded CS nanoparticles were created by membrane emulsification (ME), an environmentally benign method that produces homogeneous particles with low polydispersity index (<0.2) and high encapsulation effectiveness (~100%). To improve bioavailability and maintain antioxidant activity, a new α-tocopherol-CS conjugate was created. Better antioxidant efficacy was demonstrated by the resultant nanoparticles, which made them attractive options for drug delivery applications [43]. In previous research reported, to preserve sliced dry-cured ham for 120 days at 4°C, α-tocopherol-CS nanoparticles (TOC-CSNPs) were added to a CS/montmorillonite matrix (TOC-CSNPs/CS/MMT). TOC-CSNPs (~214 nm) were developed employing TPP-induced ionized gelation and tailored for film properties. In comparison to CS and CS/MMT films, the film displayed considerably increased radical scavenging activity and efficiently lowered peroxide and TBARS levels in ham. These findings emphasize its potential as a long-term antioxidant food packaging solution [44].

In previous research, CS, which is known for its biocompatibility and low toxicity, has been frequently converted to low-molecular-weight CS (LMWCS) to improve delivery efficiency. This study found that LMWCS can cause cytotoxicity in zebrafish liver cells, as well as developmental and systemic toxicity in larvae and adults, especially at pH <7. LMWCS disrupted cell membranes, resulting in yolk rupture in larvae and hypoxia-induced mortality in adults. Notably, its toxicity was pH-dependent and could be decreased by neutralizing negative ions, providing fresh information about LMWCS safety profiles. In the current study, biosynthesized α-tocopherol-CS bio-composite showed encouraging results in cytotoxic effect, exhibiting lower toxicity. The cytotoxic impact of investigated using the brine shrimp lethality assay, with α-tocopherol-CS bio-composite, revealed that 80% of naupalii were present at 40 and 80 μg/mL concentrations.

Limitations

In the current work, we conducted a variety of *in vitro* assays to evaluate the biological activity of the CS-α-tocopherol bio-composite. Additional investigation is needed to completely consider the anti-inflammatory properties and safety profiles of nanoparticles, antioxidant, thrombolytic, anticoagulant, cytotoxic effect, and embryonic toxicology evaluation applications. In addition, investigations, such as animal studies and clinical trials, will enhance our understanding of its effects.

CONCLUSION

Biological features of the alpha-tocopherol-chitosan bio-composite were studied, including antioxidant, antibacterial, anti-inflammatory, cytotoxicity, and embryonic toxicological. When compared to conventional controls, there were no significant differences in antioxidant, antibacterial, anti-inflammatory, and cytotoxic activities, indicating equivalent efficacy. Embryonic toxicology, as examined with zebrafish models, revealed a concentration-dependent rise in toxicity, emphasizing the importance of careful dose optimization for medicinal applications. These data indicate that the CS-α-tocopherol bio-composite has potential as a local medication delivery system, particularly for periodontal diseases. Furthermore, animal research or appropriate cell line investigations are required to demonstrate its safety and efficacy before clinical translation.

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AUTHORS' CONTRIBUTIONS

Mahalakshmi Sathishkumar: Writing–original draft, validation, methodology, investigation, formal analysis, data curation. Banuppriya Palani: Validation, methodology, and editing with review of the manuscript, data curation, and visualization. Rajeshkumar Shanmugam: Writing–review and editing, validation, supervision, methodology, investigation, data curation, conceptualization. Santhoshkumar Jayakodi: Investigation, validation, review, and editing.

DECLARATION OF COMPETING INTEREST

The authors declare no financial or personal interests that could have influenced the work presented in this study.

DATA AVAILABILITY

No data availability.

ETHICAL STATEMENT

Not applicable.

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