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Research Article

Biphasic Biological Response of *Ocimum basilicum* Loaded Hydrogels with Dose dependent Teratogenic Effects

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Abstract

Plant-derived bioactive hydrogels have attracted growing attention in regenerative medicine due to their biocompatibility and multifunctional properties. However, limited studies have explored the integration of *Ocimum basilicum* extract into crosslinked hydrogels and its effects on angiogenesis and embryonic development. This study aimed to fabricate GPTMS-crosslinked chitosan–polyvinyl alcohol (CP) hydrogels incorporated with different concentrations of *O. basilicum* extract and to evaluate their physicochemical behavior, angiogenic potential, and embryotoxicological impact using the chick CAM model. Hydrogels were prepared with extract concentrations of 0, 300, and 600 µL (CP-0, CP-3, and CP-6). Swelling and biodegradation properties were assessed under in vitro conditions. In vivo evaluation included angiogenesis quantification using the chick CAM assay, morphological and morphometric analysis of embryos, amniotic fluid biochemical profiling, and histological examination of the liver. The CP-6 hydrogel exhibited the highest swelling and faster degradation, whereas CP-3 showed stable physicochemical behavior. CAM analysis revealed that CP-3 significantly enhanced vessel formation, while CP-6 suppressed angiogenesis. Morphometric and morphological evaluations confirmed normal development in control, CP-0, and CP-3 groups, but CP-6 embryos displayed teratogenic defects, including malformed limbs, abnormal curvature, and abdominal wall defects. Amniotic fluid biomarkers indicated reduced AST (31.8 U/L) and ALT (28.5 U/L) levels in the CP-3 group, demonstrating hepatoprotective effects, whereas the CP-6 group exhibited elevated AST (48.6 U/L) and ALT (42.3 U/L) values, confirming dose-dependent hepatic and renal toxicity.. Histological findings further corroborated these outcomes, with CP-3 preserving hepatic structure and CP-6 causing vacuolation and tissue disorganization. *O. basilicum*–loaded hydrogels demonstrated a biphasic, dose-dependent response, with CP-3 showing pro-angiogenic and biocompatible potential, while CP-6 induced systemic toxicity. These findings underscore the need for dose optimization to harness herbal extract–based hydrogels for wound healing, bone regeneration, and tissue engineering applications.uminants, offering actionable targets for precision breeding in tropical agricultural economics.

Keywords: *Ocimum basilicum*, chorioallantoic membrane (CAM), hydrogels, angiogenesis, teratogenic abnormalities.

1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a complex biological process essential for embryonic development, tissue repair, and regeneration. Its

regulation plays a crucial therapeutic role—enhancement can aid ischemic and wound-healing disorders, whereas inhibition offers potential in treating neoplastic diseases [1]. Due to its central role in both physiological and pathological contexts, angiogenesis has become a key target in regenerative medicine and modern therapeutic strategies [2]. Dysregulated angiogenesis contributes to the pathogenesis of cancer, ocular disorders, and cardiovascular diseases, involving complex cellular activation, signaling cascades, and cross-talk among multiple pathways [3].

The chorioallantoic membrane (CAM) is created during avian development when the mesodermal layers of the chorion and allantois unite. This structure grows quickly, creating a rich vascular network that serves as a conduit for the exchange of waste products and gases [4]. A simple, but highly vascularised extraembryonic membrane, the chicken chorioallantoic membrane (CAM) serves a variety of purposes throughout embryonic development [4, 5]. Experts in the fields of bioengineering, development, morphology, biochemistry, transplant biology, cancer research, and drug development have expressed interest in the CAM as a reliable experimental platform for studying blood vessels [5]. A rapid, affordable, and trustworthy substitute for other animal experimental models is the CAM model [6]. The CAM eliminates the need for administrative processes to secure ethics committee approval for animal experiments and enables the rapid screening of a large number of pharmacological samples [4]. Because it has better qualities than other animal models, the chick embryo chorioallantoic membrane (CAM) model is an alternative to other experimental models. Because it lacks innervation and immune cells until the eleventh day of incubation, the CAM is painless on its own [6]. As a result, it is widely utilised to implant cancerous tumours and evaluate their propensity for metastasis and angiogenesis [4, 6]. Additionally, different biomaterials, like as collagen or hard scaffolds, can be inserted onto the CAM surface and examined primarily for their ability to cause inflammation [6]. Hydrogels, a promising class of biomaterials, can increase angiogenesis, which creates an environment that is conducive to cell growth and differentiation [7]. As a class of polymeric materials, hydrogel products have a hydrophilic structure that allows them to hold huge amounts of water in their three-dimensional networks [8]. Vinyl polymers are being researched for specific bio-based applications and are widely employed in industrial, textile, and biological applications. With their high permeability, hydrophilicity, and biocompatibility, polyvinyl alcohol (PVA) hydrogels exhibit several benefits and can be used as wound dressings. However, PVA hydrogels' inadequate mechanical qualities and lower biocompatibility compared to natural polymers render them inappropriate for use in real-world scenarios. PVA hydrogels frequently have additives added to them to improve their mechanical qualities, increase their compatibility and functioning, and broaden their range of applications [9]. Biopolymers have shown great environmental benefits due to their biodegradability. They currently constitute a significant class of materials with uses in medical as well as all other economic areas. They are used in tissue engineering, controlled drug delivery, and other fields. Due to its many remarkable qualities, including biodegradability, biocompatibility, nontoxicity, low cost, and a variety of pharmacological properties like antimicrobial, antitumor, antioxidant, antidiabetic, and immunoenhancing, chitosan is one of the natural polymers that has piqued the interest of researchers. Furthermore, it can undergo a number of structural changes due to the free amino and hydroxyl groups, producing some derivatives with various biomedical uses [10]. For improved biomedical applications, crosslinking procedures are used to increase mechanical strength, stability, and resistance to hydrolysis. Numerous cross-linkers have been studied for polymer modification [11].

The plant host system greatly benefits from the natural byproducts known as plant-based polyphenolic chemicals. These substances give wound dressings antimicrobial, antioxidant, and anti-inflammatory qualities that aid in collagen synthesis, vascularization, re-epithelization, and wound contraction [12, 13]. By providing characteristics including enhanced mechanical strength, enhanced stability, and

regulated release of bioactive substances, the addition of plant extracts to hydrogels expands their therapeutic potential. This integration promotes environmentally responsible and sustainable biomedical material practices. Hydrogels enhanced with plant extracts have been demonstrated in clinical trials to promote tissue regeneration, hasten wound healing, and treat issues including diabetic wounds [13].

Ocimum basilicum (basil) is a medicinally important herb rich in essential oils, polyphenols, flavonoids, and phenolic acids, which contribute to its strong antioxidant, antimicrobial, and anti-inflammatory properties. It has been traditionally used for managing kidney disorders and various infectious diseases [14]. The major bioactive constituents of basil, including linalool, eugenol, 1,8-cineole, methyl eugenol, and anthocyanins, are primarily responsible for its biological activities [15]. Notably, *O. basilicum* extracts have been reported to enhance angiogenesis by upregulating vascular endothelial growth factor (VEGF) gene expression in the chick chorioallantoic membrane (CAM) model, resulting in a significant increase in the number and length of newly formed vessels [16]. In contrast, studies in mice demonstrated that ethanol extracts of basil leaves at higher doses (0.21 and 0.42 mg/g body weight) inhibited VEGF expression and reduced the severity of endometriotic lesions, indicating a dose-dependent biphasic effect [17]. These findings provide a strong rationale for selecting *O. basilicum* as a natural pro-angiogenic and tissue-regenerative agent while exploring both its therapeutic and toxicological thresholds. Accordingly, the present study utilized two dose formulations (300 µL and 600 µL) to evaluate the dual angiogenic and teratogenic responses of *O. basilicum*-loaded hydrogels.

Among medicinal plants, *Ocimum basilicum* (basil) is rich in bioactive constituents that exhibit pro-angiogenic properties. However, its dose-dependent biological effects—especially its potential for both angiogenesis stimulation and toxicity—have not been systematically investigated within a hydrogel delivery system. Therefore, this study uniquely evaluates *O. basilicum*–loaded PVA/pectin/CMC hydrogels for their biphasic biological response, addressing both angiogenic potential and embryotoxicity in a single experimental framework.

Although there has been an increasing interest in bioactive hydrogels, there is a paucity of studies aimed at incorporating herbal extracts into chitosan-PVA hydrogels with the aim of assessing the intended outcomes of angiogenesis and developmental toxicity. The objective of this research was to prepare and to describe GPTMS crosslinked chitosan/PVA hydrogels with different concentrations of *Ocimum basilicum* extract, and to assess the biological activities of hydrogels by utilizing the chick CAM model.

2. Materials and methods

2.1. Materials

Chitosan (Mw = 17,103.41 g/mol; degree of deacetylation = 90.28%; viscosity = 200 cP) was purchased from Biolog GmbH (Trademark, Germany). Polyvinyl alcohol (PVA; 90% hydrolyzed, Mw = 30,000–70,000 g/mol), formic acid (Mw = 46.03 g/mol; ≥96%), glycidyloxypropyl trimethoxysilane (GPTMS; ≥98% purity), methanol, and histological staining kits were obtained from Sigma-Aldrich (USA). Fresh *Ocimum basilicum* (basil) leaves were collected from a local garden in Lahore. Whatman No. 1 filter paper (GE Healthcare, UK) was used for extract filtration, and distilled water was freshly prepared in the laboratory. Phosphate-buffered saline (PBS) tablets were purchased from Bio-World (USA) for in vitro degradation studies. A digital megapixel camera was used for CAM photography, and a MEIJI light microscope equipped with an attached digital camera (MEIJI Techno, Japan) was employed for microphotography. All chemicals were of analytical grade and used without further purification.

2.2. Methods

2.2.1 Hydrogel preparation

Chitosan–PVA hydrogels were prepared using GPTMS as a crosslinker. Briefly, 0.75 g of chitosan was dissolved in 50 mL of 2% formic acid at 60 °C under constant stirring for 2 h. In parallel, 0.25 g of polyvinyl alcohol (PVA) was dissolved in 30 mL of preheated distilled water (80 °C) with continuous stirring for 1 h. The two solutions were then combined and stirred on a hot plate for an additional 2 h. Subsequently, 25 µL of GPTMS

(pre-dissolved in 5mL of methanol) was added as a crosslinking agent, and the resulting blank hydrogel was designated as CP-0.

The herbal extract was prepared by macerating 15 g of dried *Ocimum basilicum* leaves in 100 mL of methanol for two weeks at room temperature, followed by filtration to remove solid residues. To formulate extract-loaded hydrogels, 300 µL and 600 µL of the methanolic extract were incorporated into the CP-0 hydrogel, yielding the formulations CP-3 and CP-6, respectively. All the hydrogels were casted in clean, dry petri dishes and allowed to dry at 50 °C overnight. Hydrogels were packed in polythene bags for further use.

2.2.2. Hydrogel characterization

2.2.2.1. Swelling study

Gravimetric analysis was used to assess the swelling behaviour of chitosan–PVA (CP) hydrogels. In short, sterile Petri dishes containing 5mg of each hydrogel sample were filled with 100 mL of distilled water and left to swell at room temperature. Samples were carefully taken out, blotted with filter paper to remove excess surface water, and weighed at 20-minute intervals. Until the hydrogel weight stabilised or started to decline, this process was repeated [18]. The swelling index % was calculated using the formula:

$$\text{Swelling index \%} = \left[\frac{(Wt - W0)}{W0}\right] * 100$$

where W_0 represents the initial dry weight of the hydrogel (5 mg for all samples) and Wt represents the swollen weight of the hydrogel at a given time interval. All experiments were performed in triplicate (n = 3) to ensure reproducibility, and the mean values were used for analysis.

2.2.2.2. In vitro bio-degradation of hydrogels

The in vitro biodegradation of chitosan–PVA (CP) hydrogels was evaluated using phosphate-buffered saline (PBS, pH 7.4) following the method of Azeem et al. (2023) with minor modifications [18]. Pre-weighed hydrogel samples (10 mg each) were immersed in PBS and incubated at 37 °C under static conditions. At weekly intervals, samples were removed, blotted with filter paper to eliminate excess buffer, and weighed. Fresh PBS was then replenished, and the experiment was continued for a total of 7 weeks.

2.2.3. In vivo CAM analysis

Pathogen-free fertilized White Leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from the Veterinary Research Institute (VRI), Lahore, Pakistan. Eggs were incubated at 37.5 ± 0.5 °C and 55–60% relative humidity in an automated rotatory incubator until the 10th day of embryonic development. Embryonic viability was monitored daily by candling. Viable embryos were identified by the presence of a visible vascular network, active movement, and heartbeat, whereas non-viable embryos were characterized by the absence of vessel development, cessation of heartbeat, or the appearance of a blood ring.

A total of 80 eggs of uniform size and weight (60.50 ± 5.10 g) were selected and randomly divided into four groups (n = 20 each): CP-0, CP-3, CP-6, and a control group (treated with distilled water only). On embryonic development day 5 (EDD5), a small square window was aseptically created in the shell using a sterilized minigrinder, and 6 mm² circular hydrogel discs were carefully placed onto the CAM surface in the respective groups. On EDD10, the eggs were opened using sterile forceps, and the vascular plexus of the CAM was examined. The images of the chick chorioallantoic membrane (CAM) were captured using a high-resolution digital camera and analyzed using ImageJ with the Angiogenesis Analyzer plugin. Images were converted to 8-bit grayscale, threshold adjusted, and processed to quantify the total number and length of secondary, and tertiary vessels (the software-based analysis minimized observer bias and improved the accuracy of angiogenic quantification). Any eggs exhibiting foul odor were discarded. Angiogenic responses in the treated groups were qualitatively compared with those of the control group.

2.2.3.1. Quantification of angiogenesis

Angiogenesis was quantified from CAM images by selecting regions of interest (ROIs) measuring 2 × 2 cm², with the biomaterial positioned at one of the corners. Within each ROI, the total number of newly formed vessels, including both secondary and tertiary branches, was counted manually as described by Guerra et al. (2021) [19]. Data were expressed as mean vessel counts, and results were represented graphically.

2.2.4. Amniotic fluid analysis

Amniotic fluid samples were collected on embryonic development day 10 (EDD10) from eggs containing viable embryos. After carefully removing the eggshell, a sterile syringe was inserted through the chorioallantoic and amniotic membranes to aspirate the fluid. All collections were performed under aseptic conditions to avoid contamination. Biochemical parameters of the amniotic fluid were analyzed using commercial diagnostic kits (Bayer, Germany) following the manufacturer's protocols [20].

2.2.5. Embryo recovery and morphometry

Embryos from all sets of experiments were removed from the shell and separated from their extra embryonic membranes. Yolk-free body mass was fixed in Bouin fixative for 48 hours. Embryos were photographed for estimation of any abnormality due to hydrogel exposure during development. Different motphometric parameters were estimated and their means were plotted in bar graphs [20].

2.2.6. Histology of embryos

Following fixation, tissues were dehydrated through a graded ethanol series (70%, 80%, 90%, 95%, and absolute ethanol), cleared in xylene, and embedded in paraffin wax. Paraffin blocks were sectioned at a thickness of 4–5 μm using a rotary microtome. The sections were mounted on glass slides, deparaffinized, and rehydrated through descending grades of ethanol to distilled water. Hematoxylin and eosin (H&E) staining was performed to examine general histoarchitecture, with hematoxylin used for nuclear staining and eosin for cytoplasmic and extracellular matrix components. After staining, slides were dehydrated, cleared, and mounted. Histological changes in liver development were examined under a light microscope, and representative images were captured for analysis [20].

2.3. Statistical analysis

All statistical analyses were performed using SPSS software. Data are presented as mean ± standard error of the mean (SEM). Group comparisons were conducted using one-way analysis of variance (ANOVA), followed by Duncan's and Tukey's post hoc tests where appropriate. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Swelling

Figure 1, and Table 1 (swelling values) showed a gradual rise in the swelling index % of the experimental groups up to 80 minutes followed by a small decrease. There was a slow increase in the swelling index % between 0 and 40 minutes in all the groups and no significant difference between treatments. There was a sharp rise between 40-60 minutes with CP-6 having the highest swelling index % then CP-0 and CP-3. CP-6 recorded the best swelling response at 80 minutes and CP-3 recorded relatively low values. A small decrease in swelling was noted in CP-6 and CP-3 by 100 minutes, but CP-0 was almost stationary. Comprehensively, the CP-6 group displayed the highest swelling potential, but the swelling kinetics in CP-3 was relatively slower during the assay. Our investigation is related to the results of Ibrahim et al., according to which it was noted that plant bark possesses swelling properties [21]. The observed significantly increased swelling index % in the CP-6 group indicates better hydration ability, and it might be attributed to lower cross-link density or more porosity to allow easy entry of water into it. Previous studies show that network cross-linking has a critical impact on the kinetics of water uptake and the equilibrium swelling [22]. By combining both studies, it becomes evident plant extracts have components that cause swelling. The CP-3 hydrogel, which exhibited moderate swelling rate, provided sustained swelling of *Ocimum basilicum* bioactives, promoting angiogenesis and tissue regeneration. In contrast, the higher swelling capacity observed in CP-6 may have led to a burst release of phytochemicals, including eugenol and related phenolics, which at higher concentrations could exert cytotoxic or anti-angiogenic effects.

3.2. Degradation study

The weight loss behavior of the hydrogels in PBS during six weeks showed that the experimental groups had different degradation profiles (Figure 2). First of all, each and every hydrogel showed slight weight gain in the course of the first and the second weeks, which was probably a result of water absorption and swelling.

Table 1. Showing the swelling of hydrogels. The values are represented in the form of weights of hydrogels from 0 to 100 minutes.

	CP-0	CP-3	CP-6
0	0.005±0.00	0.005±0.00	0.005±0.00
20	0.107±0.25	0.082±0.47	0.144±0.23
40	0.241±0.14	0.343±0.69	0.488±0.14
60	0.599±0.45	0.567±0.54	1.246±0.36
80	0.605±0.36	0.817±0.39	1.292±0.28
100	0.604±0.29	0.749±0.58	1.089±0.19

Note: the values are presented in the form of Mean±standard error of mean.

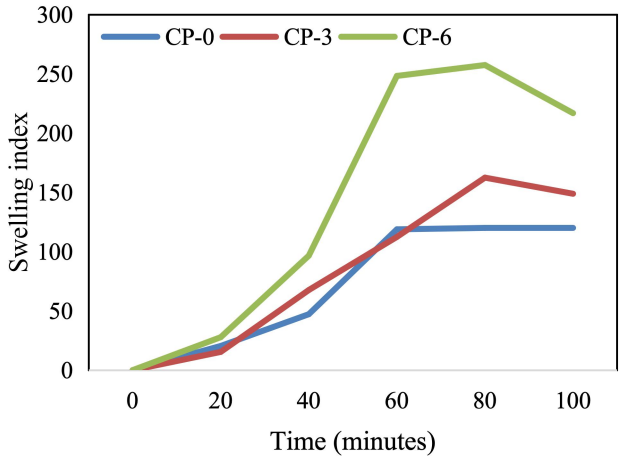

Figure 1. Swelling index % of different experimental groups (CP-0, CP-3, and CP-6) measured over 100 minutes.

The CP-3 hydrogel was the most stable during the course of the study with its weight steadily rising till week 3 and slightly decreasing until week 5 and 6. Conversely, CP-0 and CP-6 decayed more rapidly. Both groups attained maximum weights at week 2, but thereafter, weight steadily declined in both groups with full weight loss and negative values recorded at week 6. Widely, CP-3 hydrogel had a better structural stability and degradation resistance to PBS than CP-0 and CP-6 because the latter lost their mass quickly within the same duration. This effect is in line with studies that the degradation of hydrogels occurs through the hydrolytic cleavage of chains of polymers [23, 24]. According to Nwanekezie et al., plant extract contains hydroxyl and carboxyl groups [25]. Thus these groups react with the polar components and hence the hydrogel shows degradation. The CP-3 hydrogel, which exhibited a controlled degradation rate, likely provided a sustained and gradual release of *Ocimum basilicum* bioactives, thereby promoting angiogenesis and tissue regeneration.

s shown in Table 2, the residual dry weight decreased below the initial baseline (25 mg), indicating full disintegration of the hydrogel matrix. Thus, the negative readings simply reflect the mathematical outcome of the applied degradation formula once total mass loss occurs.

3.3. Qualitative and quantitative analysis of CAM vessels

The hydrogel formulations greatly influenced angiogenesis in the CAM (Figure 3, Table 3). A normal vascular plexus with moderately branched vessels was seen in the control group. The same pattern was also observed in the CP-0 group (blank hydrogel) where no significant difference was observed with control. Surprisingly, the CP-3 (300 µL extract) exhibited a strong pro-angiogenic phenotype with mass vascularization and more branching than CP-0 and control. In contrast, the CP-6 group (600 µL extract) had an apparent decrease in the development of vascularity, the vessels were sparse and poorly branched. There was a significant increase in the number of vessels in CP-3 that was not observed in any other group ($p < 0.05$), which confirmed a strong pro-angiogenic effect. Meanwhile, CP-6 exhibited a significant decrease of secondary and tertiary vessels indicating an inhibitory effect (Table 1). Our study is supported by previous studies, where alcoholic *O. basilicum* leaf extract stimulated vessel formation in a CAM based study [26]. The enhanced angiogenic response observed in the CP-3 group may be attributed to the activation of pro-angiogenic signaling pathways such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). The flavonoids and phenolic acids in *Ocimum basilicum*, particularly eugenol, have been reported to upregulate VEGF expression and stimulate endothelial cell proliferation and migration, facilitating neovascularization. This mechanism aligns with previous study [16].

Table 2. In vitro biodegradation profile of *Ocimum basilicum*–loaded PVA/pectin/CMC hydrogels (CP-0, CP-3, and CP-6) over six days in PBS (pH 7.4) at 37 °C.Each hydrogel initially weighed 25 mg. A gradual decrease in dry weight was observed over time, indicating progressive degradation.

	CP-0	CP-3	CP-6
Day 0	25±0.00	25±0.00	25±0.00
1	35±0.59	38±1.11	32±0.13
2	37±0.69	40±1.25	33±0.21
3	31±1.22	41±2.12	29±0.95
4	24±1.53	41±2.13	25±0.27
5	21±0.98	40±1.65	20±1.02
6	19±0.52	38±0.45	18±2.45

Note: Values are presented as mean±SEM.

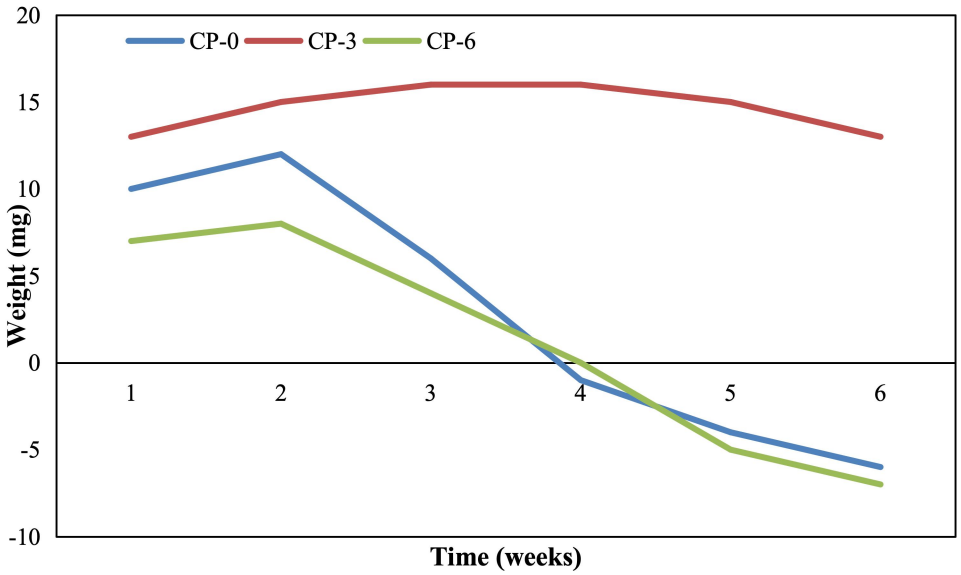

Figure 2. Degradation profile of hydrogels (CP-0, CP-3, and CP-6) in PBS over six weeks.

Nevertheless, the greatest extract concentration (CP-6) significantly inhibited vascularization as can be paralleled in another study where mice exposed to 0.21 mg/g-BW and 0.42 mg/g-BW of basil leaf ethanol extract there was effective decrease in VEGF expression and limit in severity of endometriosis lesions [17]. These observations indicate the multifaceted pharmacological characteristics of herbal extracts, in which constituents such as flavonoids, terpenoids, and polyphenols can have angiogenic stimulatory effects at effective doses but angiogenic inhibitory effects at high doses or teratogenic effects at high doses.

At the higher dose (CP-6), the hydrogels exhibited reduced angiogenesis and signs of hepatic and renal stress, which may be attributed to the cytotoxic effects of excessive *Ocimum basilicum* constituents such as eugenol. While eugenol is known for its potent antioxidant and anti-inflammatory properties at low concentrations, higher doses can shift its action toward pro-oxidant and cytotoxic mechanisms. Previous

studies have demonstrated that eugenol induces apoptosis through reactive oxygen species (ROS) generation, mitochondrial permeability transition (MPT), downregulation of the anti-apoptotic protein Bcl-2, and cytochrome-c release, leading to programmed cell death [27]. Similarly, Nejad et al. (2019) reported that eugenol caused significant DNA damage at higher concentrations compared with untreated controls [28]. These findings suggest that the cytotoxic and teratogenic effects observed in the CP-6 group may result from ROS-mediated oxidative stress and apoptotic pathways triggered by euglenol overexposure.

3.4. Analysis of liver and kidney biomarkers from amniotic fluid

Amniotic fluid biochemical analyses showed that there were significant, dose-dependent differences in liver and kidney biomarkers within treatment groups (Table 2). There was no difference in the extent of total bilirubin in any of the groups. On the contrary, hepatic enzymes showed significant changes. The CP-3 group had lower AST (31.8 ± 1.06 U/L) and ALT (29 ± 0.70 U/L) than the control, which indicates a hepatoprotective effect at this concentration. On the other hand, in CP-6, AST (48.6 ± 1.07 U/L), ALT (46 ± 1.93 U/L), and ALP (304.4 ± 0.92 U/L) were significantly elevated over all other groups and are indicative of hepatic dysfunction. Effects of treatment were also manifested in renal biomarkers. Although creatinine and urea concentration in the control and CP-0 and 3 maintained a similar range, CP-6 exhibited a substantial increase in creatinine (2.26 ± 0.08 mg/dL) and urea (48.8 ± 1.42 mg/dL), which is indicative of stress in the renal system. Collectively, these findings suggest that the CP-3 (300 µL) extract preparation was tolerable and possibly protective, and the higher concentration (CP-6) caused hepatotoxic and nephrotoxic outcomes in growing chick embryos. Amniotic fluid is a prominent factor in the health and development of the fetus [29]. Thus, our data were consistent with recent studies of avian embryotoxicity, in which amniotic fluid biomarkers were useful predictors of systemic toxicity. As an example, in a 2024 study examining the toxicology effects of titanium dioxide nanoparticles on the embryo of chickens, tissue damage and metabolic derangement were accompanied by significant changes in amniotic fluid AST, ALT, ALP, urea, and creatinine, which are sensitive biomarkers that can be used to determine the organ functionality of embryos [30].

Amniotic fluid biomarkers such as ALT, AST, ALP, and creatinine serve as reliable indicators of hepatic and renal function in developing embryos [31].

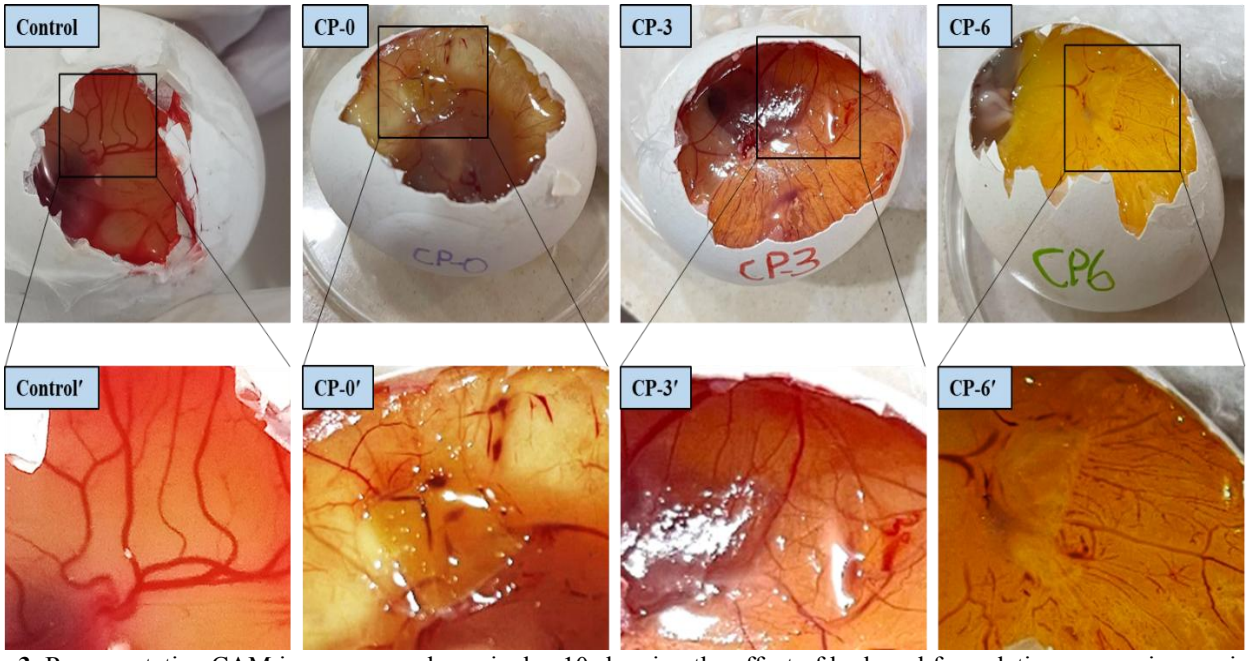

Figure 3. Representative CAM images on embryonic day 10 showing the effect of hydrogel formulations on angiogenesis. CP-3 (300 µL extract) exhibited the highest vascular density and branching compared to control and CP-0, while CP-6 (600 µL extract) showed reduced vessel formation.

Table 3. Mean number of secondary and tertiary blood vessels in CAM of chick embryos on embryonic development day 10 (EDD10) after treatment with different hydrogel formulations.

Groups	Secondary blood vessels	Tertiary blood vessels
Control	$6.56^{c}\pm0.48$	$16.80^{c}\pm0.34$
CP-0	$5.79^{b}\pm1.97$	$17.0^{b}\pm0.17$
CP-3	$14.56^{a}\pm0.99$	$30.40^{a}\pm1.33$
CP-6	$5.11^{d}\pm0.12$	$12.0^{d}\pm0.63$

Note: Values represent mean ± SEM (alphabets represent a significant difference among groups). Statistical comparisons were performed using one-way ANOVA followed by post hoc tests, with $p < 0.05$ considered significant.

Table 4. Biochemical parameters of amniotic fluid from chick embryos on embryonic development day 10 (EDD10) following treatment with different hydrogel formulations.

Parameters	Control	CP-0	CP-3	CP-6
Bilirubin total (mg/dl)	$0.52^{a}\pm0.19$	$0.64^{a}\pm0.15$	$0.56^{a}\pm0.18$	$0.62^{a}\pm0.23$
AST (U/L)	$38.6^{b}\pm1.92$	$39.6^{b}\pm1.27$	$31.8^{c}\pm1.06$	$48.6^{a}\pm1.07$
ALP (U/L)	$234.2^{b}\pm2.96$	$240.6^{b}\pm1.47$	$240.4^{b}\pm0.92$	$304.4^{a}\pm0.92$
ALT (U/L)	$32^{b}\pm1.86$	$31^{b}\pm2.86$	$29^{c}\pm0.70$	$46^{a}\pm1.93$
Creatinine (mg/dL)	$0.74^{b}\pm0.19$	$0.50^{b}\pm0.38$	$0.98^{b}\pm0.02$	$2.26^{a}\pm0.08$
Urea (mg/dL)	$36.6^{bc}\pm1.07$	$35.9^{c}\pm1.81$	$38.2^{b}\pm2.15$	$48.8^{a}\pm1.42$

Note: Values are expressed as mean ± SEM (n = 20). Different superscript letters within a row indicate statistically significant differences among groups (one-way ANOVA with post hoc tests, $p < 0.05$).

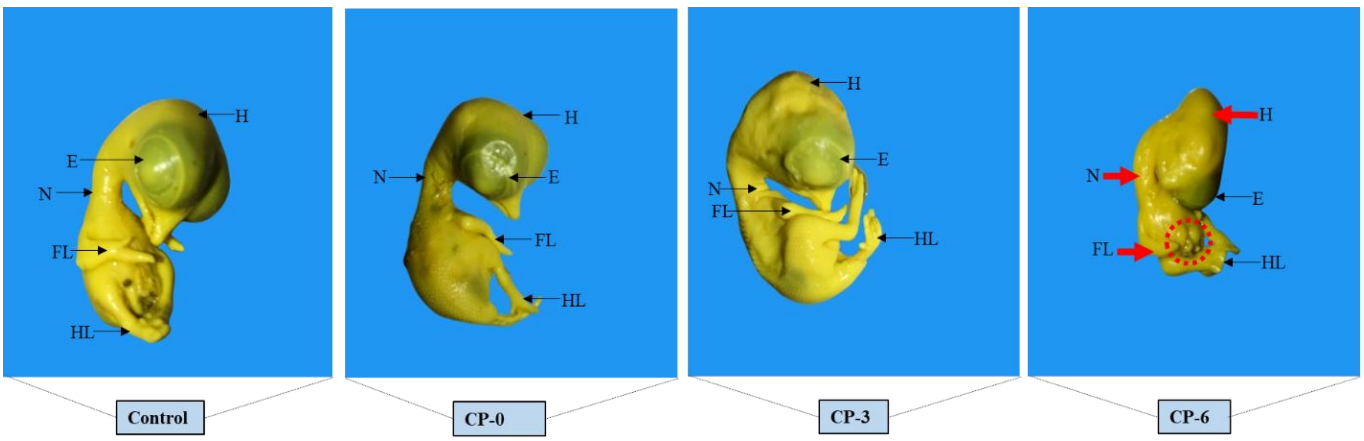

Figure 4. Representative chick embryos on EDD10 showing the effects of hydrogel formulations on morphology. In the CP-6 group red dotted circle in the abdomen shows defective abdominal wall. Red arrows show defects in neck, head, and forelimb. Note: head (H), eye (E), neck (N), forelimbs (FL), and hindlimbs (HL).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are key hepatic enzymes reflecting hepatocellular integrity, with elevated levels typically indicating liver injury or metabolic stress. Alkaline phosphatase (ALP) plays a role in tissue remodeling and bone mineralization, and its fluctuation may reflect altered developmental metabolism. Similarly, creatinine and urea are important renal biomarkers whose elevated concentrations correspond to impaired kidney filtration or developmental nephrotoxicity. In this context, the reduced AST and ALT levels observed in the CP-3 group indicate hepatoprotection, whereas the increased creatinine and urea levels in CP-6 suggest potential renal stress at higher doses of the extract.

3.5. Morphology and morphometric analysis

Morphological analysis of chick embryos on EDD10 showed that there were distinct morphological variations between the treatment groups (Figure 4). The control and CP-0 (blank hydrogel) groups exhibited normal embryogenesis including well-formed head (H), eye (E), neck (N), forelimbs (FL) and hindlimbs (HL) and no observed malformation. Also, the CP-3 group (300 µL extract) exhibited normal morphology, the embryos resembling control, indicating that there was no teratogenic effect of this concentration. By contrast, the CP-6 group (600 µL extract) embryos showed a severe teratogenic phenotype with defects in the limbs, abnormal head and neck curvature, and abdominal wall defect. These data point out that *Ocimum basilicum* extract exerted a dose-dependent effect, with the lower-concentrated solution (300 µL) being conducive to normal development and angiogenesis and the higher-concentrated solution (600 µL) leading to an effect on embryogenesis and structural defects. Our results are consistent with the results that plant-derived extracts may have bi-phasic effects on embryo development, whereby, at lower doses, they are tolerated or advantageous, at higher concentrations they cause toxicity and morphometric defects. In one example; Abebe et al., showed that a high dose of exposure to a medicinal plant extract in the rat embryo resulted in a shorter crown-rump length, deformed digits and low morphological scores- important signs of growth retardation and teratogenicity [32].

Morphometric analysis of chick embryos showed that great differences between treatment groups were observed (Table 5). CP-3 group had the greatest body length (28.0 ± 1.24 mm), extended hindlimb length (6.56 ± 0.23 mm), and general normal development, which showed remarkable uplift over the control and CP-0 ($p < 0.05$). On the contrary, the CP-6 group had abnormal growth forms with disproportionate high body weight (5.06 ± 1.19 g), short body length (21.2 ± 0.74 mm), short limbs, and long heads, which are typical of teratogenic effects. The average crown-rump length of 9-10-day-old chick embryos has been reported to range between 25 and 28 mm, with normal development of limb buds and head structures [31]. These baseline values correspond closely to our control group (25 mm), supporting that the reduced body length recorded in the CP-6 group (21.2 mm) represents developmental delay and teratogenic effects rather than normal variation. There was similarity in the morphometric values in control and CP-0 groups and no significant differences. These results affirm that *Ocimum basilicum* extract of 300 µL could promote normal embryonic growth, but 600 µL provoked developmental defects.

Table 5. Morphometric parameters of chick embryos on embryonic development day 10 (EDD10) following treatment with different hydrogel formulations.

Parameters	Control	CP-0	CP-3	CP-6
Body Weight (g)	0.79^{b}±0.29	0.71^{b}±0.10	0.87^{a}±0.21	5.06^{c}±1.19
Body length (mm)	25.0^{b}±0.47	23.4^{b}±1.74	28.0^{a}±1.24	21.2^{c}±0.74
Neck size (mm)	3.68^{ab}±0.44	3.00^{c}±0.29	3.14^{b}±0.54	3.34^{a}±0.15
Forelimb length (mm)	4.92^{a}±0.33	4.39^{a}±0.33	5.38^{a}±0.31	2.88^{b}±0.40
Hindlimb length (mm)	6.28^{a}±0.25	5.54^{b}±0.15	6.56^{a}±0.23	3.30^{c}±0.24
Eye circumference (mm^2)	4.52^{a}±0.73	5.48^{a}±0.12	4.78^{a} ±0.05	3.19^{b}±0.10
Head circumference (mm^2)	5.32^{b}±0.08	7.54^{a}±0.10	7.48^{a}±0.08	4.28^{c}±0.81

Note: Values are expressed as mean ± SEM (n = 20). Different superscript letters within a row indicate statistically significant differences among groups (one-way ANOVA with post hoc tests, $p < 0.05$).

3.6. Liver histology

Liver sections of embryos were examined histologically and showed that the groups had clear structural differences (Figure 5). The hepatic cords in the control group were set in a normal manner, accompanied by observable hepatocytes (H), hepatocyte nuclei (Hn), central vein (CV), and sinuoidal spaces (SD). The architecture was less compact in CP-0, and hepatocytes were loosely organized with widened sinuoids.

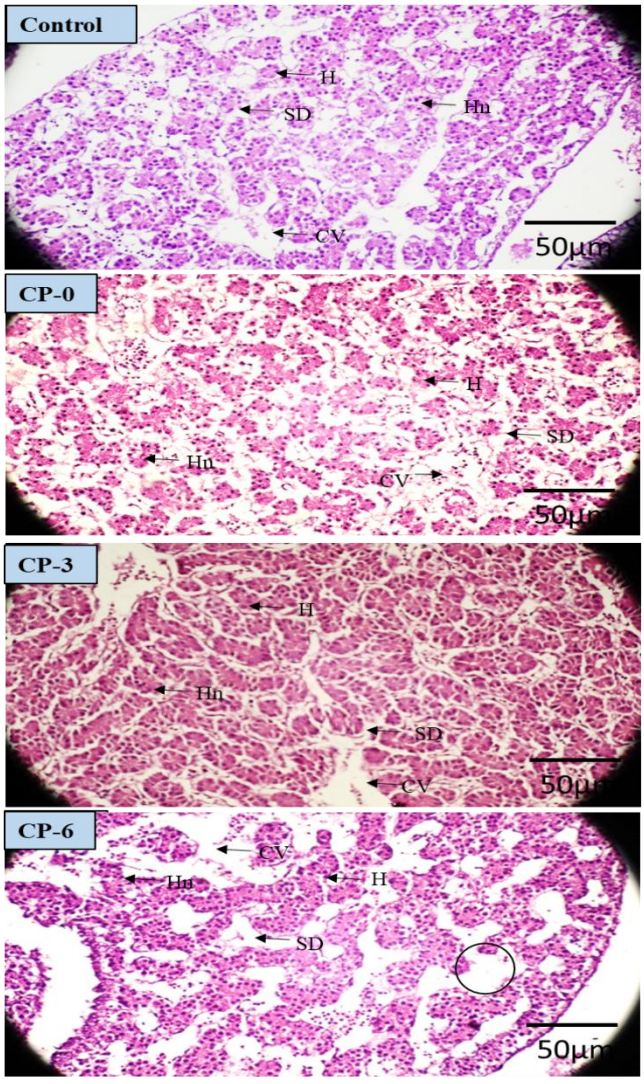

Figure 5. Histological sections of embryonic liver at 40X magnification (H&E staining, 50 µm scale bar). Circular area in CP-6 group shows vacuolation.

Interestingly, in CP-3 hepatic regions were more regular, organized, the arrangement of hepatocytes was compact, and sinuoids were better defined than in the control, indicating an improved development. In contrast, CP-6 was characterized by irregular central vein formation, disoriented hepatocyte position, disordered cell structure, and significantly expanded sinuoidal cavities, which showed that hepatic tissue organization was disrupted. Our results are consistent with the xenobiotic-induced hepatic damage of a high degree reported in the study by Islam et al., including vacuolation and disturbed tissue structure [30].

4. Conclusion

This study demonstrates that *Ocimum basilicum*–impregnated chitosan–PVA hydrogels exhibit dose-dependent biological effects in the chick embryo model. The lower dose (CP-3; 300 µL extract) promoted angiogenesis and maintained hepatic and renal integrity, supporting its potential as a biocompatible, pro-angiogenic scaffold. In contrast, the higher dose (CP-6; 600 µL extract) induced developmental and hepatic toxicity, confirming the biphasic nature of *O. basilicum* bioactivity. However, since the CAM model represents a preliminary non-mammalian system, further validation in mammalian models is essential to confirm these effects and to elucidate molecular mechanisms before any translational or clinical applications can be considered. Further validation in mammalian systems and molecular investigations into VEGF-mediated pathways are essential to confirm efficacy, define safe concentrations, and guide translational applications in regenerative medicine.

Author contributions

A.Z. conceived the idea, wrote the original manuscript, supervised the research, formatted the figures, and prepared the tables. R.H. contributed to writing and editing. S.A., A.A., M.I., M.A., and M.A.A. assisted in manuscript preparation and data organization. All authors have read and agreed to the published version of the manuscript.

Ethical approval

Not applicable

Conflicts of Interest

The authors report no conflicts of interest.

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Data availability statement

The data presented in this study are available on request from the corresponding author.

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