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Histochemical Analysis of the Effects of *Gliricidia sepium* Floral Extract on Glycogen, Protein, and Lipid Distribution in Early Chick Embryo

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Abstract: This study investigates the histochemical impact of *Gliricidia sepium* floral extract on the distribution of glycogen, protein, and lipid in early chick embryos. Using specific staining techniques, embryos were examined at 24, 48, and 72 hours after incubation periods following *in Ovo* administration of two concentrations (1 mg/50 ml and 1 mg/100 ml) of the extract. Results indicate concentration-dependent variations in biomolecule distribution, with higher extract concentrations inducing notable morphological changes, including distortions in embryonic tissues. The findings suggest that *Gliricidia sepium* may modulate embryonic biomolecular content and influence key developmental processes, thereby warranting further investigation into its bioactive constituents and their mechanism of action.

Keywords: *Gliricidia sepium*, Chick embryo, Histochemistry, Glycogen, Protein, Lipid, Embryonic development, Concentration-dependent effect

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Introduction

Histochemistry, a specialized discipline within biochemistry, focuses on the visualization and localization of chemical components within biological tissues and cells (Ahmed *et al*., 2020). It serves as a powerful tool to explore the spatial distribution of biomolecules, offering crucial insights into their physiological and pathological roles. Histochemical techniques are particularly valuable in studying embryonic development, as they allow researchers to examine dynamic changes in the localization and abundance of proteins, glycogen, and lipids during critical developmental stages (Galli *et al*., 2004).

During embryogenesis, the regulation and distribution of biomolecules play pivotal roles in cell differentiation, tissue morphogenesis, and organ formation. Glycogen and lipids, for example, are not only key energy reserves but also serve

structural and signaling functions essential for embryonic growth and development (Liu *et al*., 2021). Proteins, acting as enzymes, structural components, and signaling molecules, are central to various processes, including cell proliferation, neural development, and immune function (Galli *et al*., 2004; Jamiyansuren and Khurelbaatar, 2017).

The chick embryo is an established and highly informative model for developmental studies due to its accessibility, external development, and rapid, well-characterized growth stages (Romanoff, 1960). These features enable precise experimental manipulation and real time observation of developmental changes, making it ideal for evaluating the impact of exogenous compounds on embryogenesis.

In recent years, increasing attention has been directed toward the biological effects of plant-derived extracts, especially in the context of developmental biology and toxicology. *Gliricidia sepium*, a nitrogen-fixing tree native to Central America and widely cultivated in tropical regions, has notable agroforestry value due to its rapid growth, soil-enriching properties, and use as fodder and fuel (Mohammed *et al*., 2020; Gavilánez-Buñay *et al*., 2023). However, certain phytochemicals present in *G. sepium*, such as mimosine and cyanogenic glycosides, have been associated with adverse physiological effects, including hair loss, thyroid dysfunction, and cyanide poisoning in animals (Rahman *et al*., 2019). Despite its widespread use, the potential embryotoxic or teratogenic effects of *G. sepium* floral extracts have not been adequately explored (Aulanni'am *et al*., 2021)

This study aims to investigate the histochemical impact of *Gliricidia sepium* flower extract on chick embryo development, with a specific focus on the distribution and quantification of key biomolecule proteins, glycogen, and lipids in embryonic tissues. By analyzing these changes, we seek to understand the developmental alterations induced by this plant extract and contribute to the broader field of environmental and botanical toxicology.

Materials and Methods

Collection and preparation of Gliricidia floral extract

Fresh flowers of *Gliricidia sepium* were collected from the campus of Fergusson College, Pune, India. The collected flowers were air-dried at room temperature for one week and ground into a fine powder using a mortar and pestle. Soxhlet extraction was carried out using 80% ethanol (Cherian, 2019) to prepare the floral extract. Specifically, 3.283 g of powdered material was immersed in ethanol and left to soak for one week. The choice of ethanol was due to its efficiency in eluting polar compounds (Handa *et al.*, 2008; Azra Tariq *et al*., 2016). Post-extraction, the solution was filtered using Whatman filter paper, and the filtrate was concentrated using a rotary evaporator at 80°C. The resulting viscous extract was stored in labeled vials for further use (Gavilánez-Buñay *et al*., 2023).

Acquisition and incubation of fertilized chicken eggs

Fertilized eggs (0 h stage) of *Gallus domesticus* (White Leghorn strain) were procured from Venkateshwara Hatcheries Pvt. Ltd., Pune, India. Eggs were sterilized by washing with distilled water and wiping with 70% ethanol, then incubated at 37.5°C and 70–80% relative humidity in a BOD incubator for 24, 48, and 72 h. Embryonic stages were identified using Hamburger and Hamilton's developmental stages (Hamburger and Hamilton, 1992; Bellairs and Osmond, 2005).

Preparation of extract solutions

Two concentrations of *Gliricidia* floral extract were prepared: 1 mg/ml in 50 ml (Solution A) and 1 mg/ml in 100 ml (Solution B) of solvent. Solutions were labeled accordingly (Kumar *et al*., 2014).

Experimental grouping and egg injection protocol

For each time point (24, 48, and 72 h), four eggs were selected: two for protein and glycogen analysis and two for lipid analysis. Each pair

included one egg injected with Solution A and the other with Solution B. A total of 12 eggs were used for the experimental study (Fauzia *et al*., 2018). 12 eggs were also used for control without any treatment.

Injection was performed into the egg yolk using a sterile syringe with a fine needle, delivering 1 ml of the respective extract solution. Eggs were incubated under standard conditions until respective endpoints (Ashworth *et al*., 2009).

Tissue fixation

At each time point, embryos were dissected using sterile instruments in a clean environment. After removing the embryos, they were immersed in fixative solutions:

- *For protein and glycogen*: Carnoy's fixative (60 ml absolute alcohol, 30 ml chloroform, 10 ml glacial acetic acid) (Panja *et al*., 2007; Agrawal and Jose, 2019).
- *For lipids*: 5% mercuric chloride with 2.5 g potassium dichromate per 100 ml, pH adjusted to 2.5 using HCl (Raviola and Raviola, 1953).

Embryos were fixed for 24 h (protein/glycogen) or 4 h at 4°C (lipids), then rinsed with PBS (Ruth and Osmond, 2005; Al-Sabawy *et al*., 2021).

Tissue processing and staining

Fixed tissues were dehydrated using graded ethanol (70%, 95%, 100%) and cleared in xylene. Samples were infiltrated with melted paraffin wax (55–60°C), embedded, cooled, and trimmed for sectioning (Luna,1968). Tissue sections of 7 µm thickness were obtained using a rotary microtome and mounted onto glass slides. Slides were air-dried for 24 h. Slides were deparaffinized in xylene and rehydrated through graded ethanol to distilled water, each step lasting 3 min (Luna *et al*., n.d). The sections were stained as follow:

- *Protein Staining*: Bromophenol blue staining for 1.5–2 h, followed by 1% acetic acid, tertiary butyl alcohol wash, xylene clearing, and DPX mounting. Proteins stained blue (Nowak-Sliwinska *et al*., 2014).
- *Glycogen Staining*: Best's carmine staining after alum hematoxylin counterstaining; sections were washed with 90% and absolute alcohol, cleared in xylene, and mounted. Glycogen stained magenta (Ansari *et al*., 2025).
- *Lipid Staining*: Sudan Black B in ethylene glycol, differentiated with 85% ethylene glycol, washed, and mounted in glycerin jelly. Lipids appeared black (Rolle, 1965).

Results and Discussion

Protein, glycogen, and lipid content were histochemically analyzed in chick embryos treated with *Gliricidia sepium* extract at two concentrations over 24, 48, and 72 h and compared with controls.

Protein Localization

Mercury bromophenol blue staining indicated a progressive increase in protein deposition in treated embryos over time. At 24 h, mild blue staining was noted in control embryos, primarily in the neural tube. In contrast, both treated groups showed increased intensity with the 1 mg/50 ml group displaying more prominent protein presence in the somites and cardiac tissue (Fig. 1). These observations suggest enhanced protein synthesis, potentially stimulated by phytochemicals such as alkaloids and flavonoids in *G. sepium* (Couto *et al*., 2022; Wafaey *et al*., 2025).

At 48 and 72 h, treated embryos continued to exhibit more intense staining in areas like the optic vesicle, truncus arteriosus, and neural crest compared to controls. These findings support other reports which describe that bioactive plant compounds can promote embryonic cell proliferation and differentiation (Nowak-Sliwinska *et al*., 2014; Naz *et al*., 2022).

Glycogen Accumulation

Best's Carmine staining showed minimal magenta color in control embryos at 24 h (Trott, 1961). However, treated embryos, particularly those

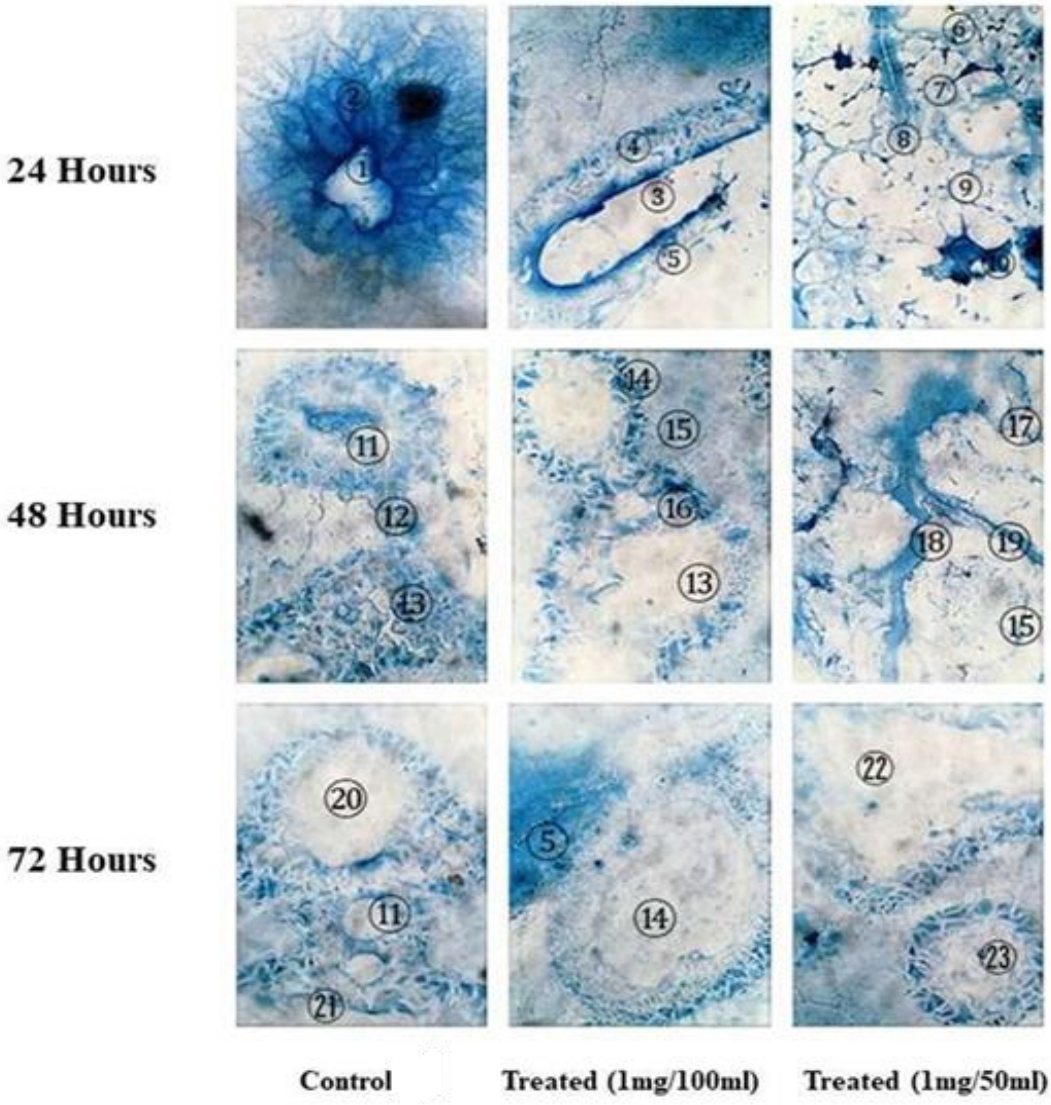

Fig. 1: Protein: control vs treated. 91) Blood island, (2) Blood vessel, (3) Neural groove, (4) Neural fold, (5) Foregut, (6) Ectoderm, (7) Neural tube, (8) Head mesoderm, (9) Subcephalic cavity (amnion region), (10) Area opaca (Dark), (11) optic vesicle, (12) Myencephalon, (13) Chorda, (14) Heart Formation, (15) Pharynx, (16) Dorsal aorta arches, (17) Ventricle, (18) Dorsal aorta arches, (19) Pericardial cavity, (20) Mesencephalon, (21) Diencephalon, (22) Atrium, (23) Truncus atrium.

exposed to the 1 mg/50 ml extract, showed higher glycogen accumulation in the neural tube and heart region (Fig 2). At 48 h, this trend continued with glycogen prominently localized in the gut and aortic arches. By 72 h, treated groups displayed dense staining in the lung bud and liver primordium.

These results suggest an extract-induced increase in metabolic activity, which may be linked to enhanced glucose uptake and storage via stimulation of glycogen synthase pathways (Gupta *et al*., 2011; Kumar *et al*., 2021). Similar glycogen enhancement by herbal extracts has been reported in chick embryo models and diabetic rats (Gupta *et al*., 2011; Kumar *et al*., 2021).

Lipid Distribution

Sudan Black B staining revealed increased lipid

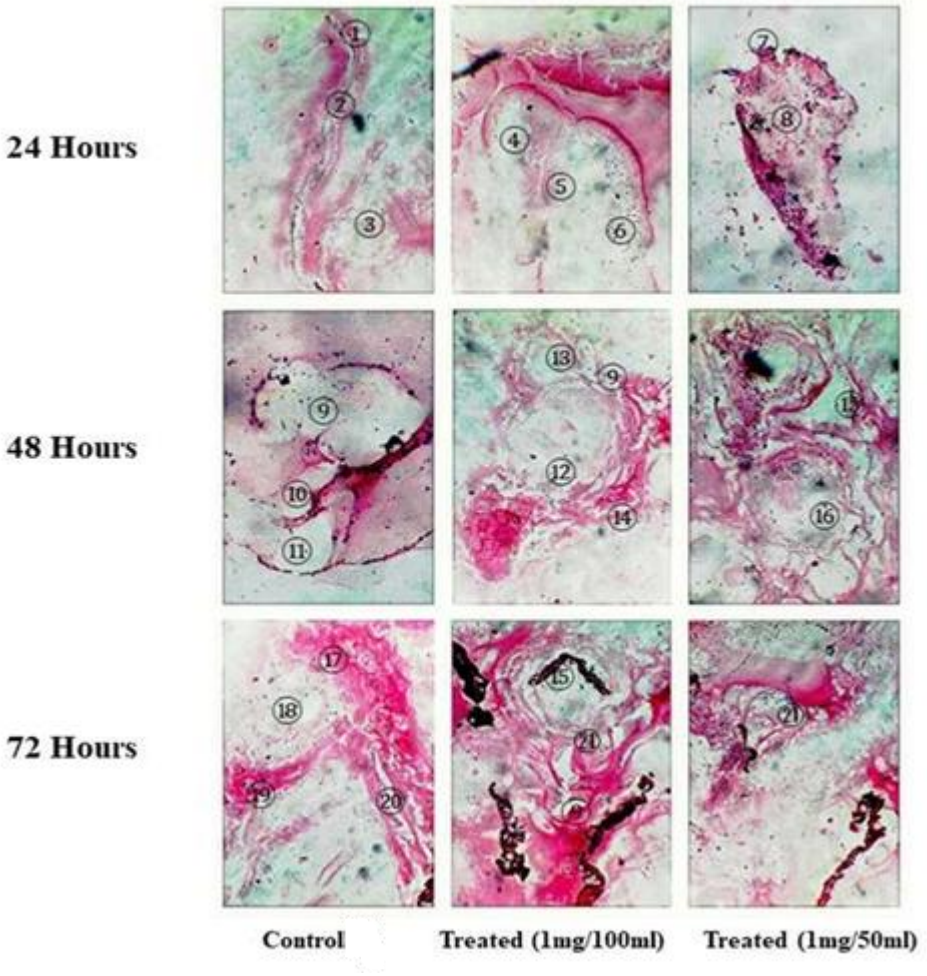

Fig. 2: Glycogen: control vs treated. (1)Head mesoderm, (2) Endoderm, (3) Blood vessel, (4) Endocardium, (5) Mesocardium, (6) Epimyocardium, (7) Cranial neuropore, (8) Subcephalic cavity, (9) Dorsal aortic arches, (10) Pharynx, (11) Heart Formation, (12) Spine, (13) Chorda, (14) Dermatome, (15) Atrium, (16) Ventricle, (17) Optic bulb, (18) Lens, (19) Epiphyse, (20) Neural tube, (21) Foregut with folding of lung bud.

accumulation in treated embryos compared to controls (Fig. 3). At 24 h, slight lipid presence was observed in the treated embryos' neural regions. By 48 h, lipid-rich areas were evident in the optic vesicles and developing myotomes (Ding *et al*., 2023). At 72 h, significant staining was seen in the diencephalon, telencephalon, and liver.

The higher lipid content in treated embryos indicates enhanced membrane formation and energy storage, crucial during organogenesis (Donaldson, 1981; Ashworth *et al*., 2009). These findings are in agreement with previous studies showing plant-derived compounds influencing lipid droplet metabolism and cell membrane stability (Liu *et al*., 2020; Sun *et al*., 2020).

Comparative Concentration Analysis

In nearly all cases, the 1 mg/50 ml concentration yielded more pronounced histochemical changes than the 1 mg/100 ml concentration. While higher concentrations appeared more effective in stimulating biomolecule accumulation, they also introduced slight morphological distortion in some embryos, indicating a possible threshold for safe exposure (Herrera-Moro *et al*., 2023).

Conclusion

This study demonstrates that *Gliricidia sepium* floral extract affects early chick embryogenesis by altering the histochemical profiles of protein, glycogen, and lipid. Embryos treated with both 1 mg/50 ml and 1 mg/100 ml concentrations showed enhanced biomolecule accumulation in a

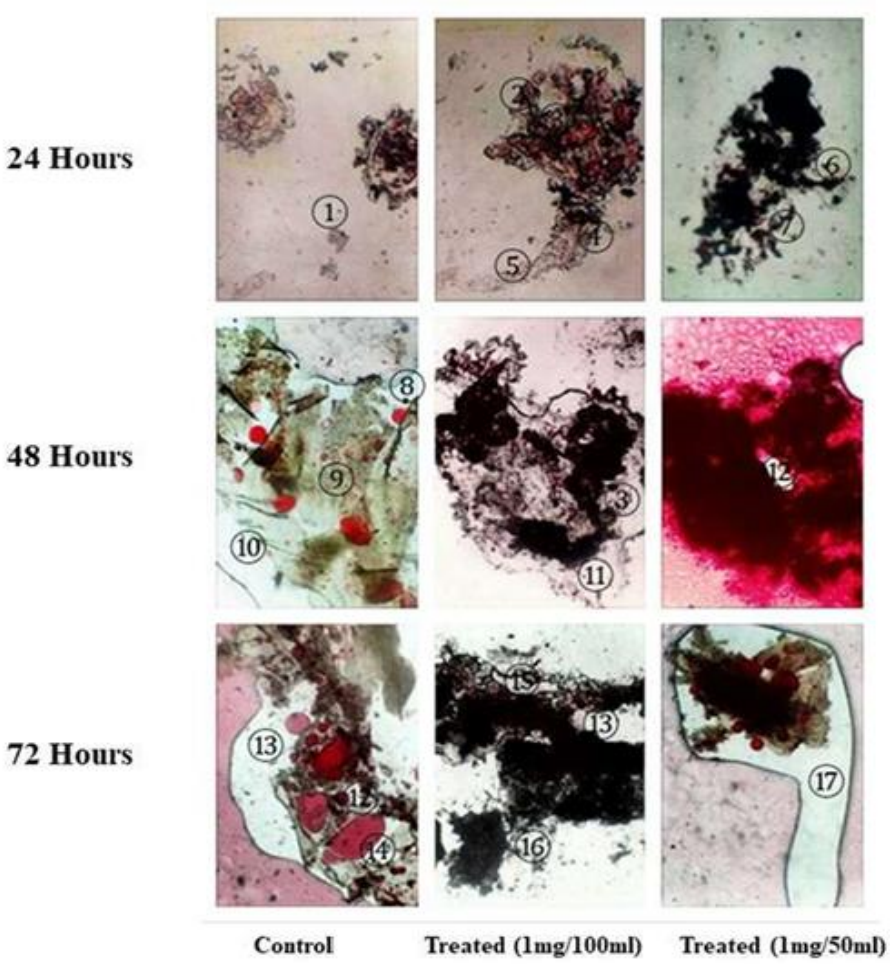

Fig. 3: Lipid: control vs treated. (1) Blood islands, (2) Neural Fold, (3) Foregut, (4) chorda, (5) Primitive groove, (6) Head mesoderm, (7) Subcephalic cavity, (8) Endoderm, (9)Head mesoderm, (10) Ectoderm, (11) Dermatome, (12)Optic vesicle, (13) Dicephalon, (14) Lens, (15) Central hemisphere of telencephalon, (16) Olfactory groove, (17) Atrium.

dose- and stage-dependent manner. Higher concentrations led to stronger staining and elevated biomolecular presence but were also associated with mild structural distortion, suggesting potential toxicity at higher doses. These findings highlight the extract's bioactive potential in modulating embryonic metabolism and underscore the importance of dosage control. Future studies should focus on identifying active compounds, exploring their molecular mechanisms, and assessing the long-term developmental impacts *in vivo*.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the Department of Zoology, Fergusson College, Pune, India.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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