

VOLUME 9

ISSUE 1

2023

ISSN 2454 – 3055



**INTERNATIONAL
JOURNAL OF
ZOOLOGICAL
INVESTIGATIONS**

*Forum for Biological and
Environmental Sciences*

Published by Saran Publications, India



Histochemical Study on the Effect of *Tridax procumbens* (L.) Leaf Extract on Glycogen and Lipid Content in Chick Embryo Vitelline Blood Vessels

Pendharkar Kishor Dhanji

Department of Zoology, Fergusson College (Autonomous), Pune 4, India

Received: 14th December, 2022; **Accepted:** 6th January, 2023; **Published online:** 24th January, 2023

<https://doi.org/10.33745/ijzi.2023.v09i01.013>

Abstract: Wound healing is a repair process at cellular and tissue level. It begins immediately after an injury to the epidermal layer. The repair process includes organized cellular proliferation, and remodeling mechanisms. There are large numbers of cell types including lymphocytes, fibroblasts, and endothelial cells. Traditional herbal medicines are used for wound healing. A plant *Tridax procumbens* (L.) is very commonly used as herbal medicine in healing wounds. The medicinal use of *Tridax* has inspired the author for histochemical studies which would be providing important information. This histochemical study reveals and compares content of glycogen and lipids in the blood vessels in control and *Tridax* leaf extract treated samples. The effect of *Tridax* leaf extract was studied on carbohydrate (glycogen) and lipid by histochemistry on 48 h and 96 h chick embryo vitelline blood vessels. These biomolecules are large and complex molecules that play many critical roles in the body. For histochemical work it is important to have ways of demonstrating these bio molecules.

The histochemistry of Glycogen in developing blood vessels was studied using alum hematoxylin. Sudan black staining is used to detect the lipid content. The application of histochemical study and tests for quantitative estimation is discussed in connection with the characterization of these biomolecules. The objective of this histochemical study was to evaluate the effect of *Tridax procumbens* leaf extract for the characterization of these biomolecules with respect to healing properties in the early chick embryo.

Keywords: Chick embryo, Histochemistry, Glycogen, Lipid, Blood vessel, Vascular mesoderm, *Tridax procumbens*

Citation: Pendharkar Kishor Dhanji: Histochemical study on the effect of *Tridax procumbens* (L.) leaf extract on glycogen and lipid content in chick embryo vitelline blood vessels. Intern. J. Zool. Invest. 9(1): 126-132, 2023.

<https://doi.org/10.33745/ijzi.2023.v09i01.013>



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Introduction

Histochemistry has brought together knowledge of cell structure, organization pattern and biochemical constituents and defines the function of tissue as well (Elledar and Lojda, 1984; Spicer, 1993). It has localized biomolecules of specific biological activity. Histochemistry has contributed

to detecting the function of organs and system level by detecting heterogeneity in the content of the biomolecules like proteins, carbohydrates, and lipids. On the other hand, histochemistry has suggested a possible role for the constituent related to that of the structure (Campbell and

Gibson, 1970). Histochemical examination provides information about composition of biomolecules which is obtained by the chemical techniques (Postma *et al.*, 1978). Glycogen and lipids perform vital roles in every aspect of life processes. These molecules are encoded in DNA from where transcribed and translated in the specific cellular organelles and translocated to their location. Some sugars and fats play structural roles and are part of cytoskeleton and cell membrane. Therefore, analysis of histochemical localization of glycogen and lipids and their quantification are important aspect of study in histochemical research. Glycogen is a large and branched polysaccharide that is the main stored form of glucose in animals. Glycogen is as an important energy reservoir; when energy is required by the body, glycogen is broken down into glucose, which then enters the glycolytic pathway and is released into the blood (Eram Fauzia *et al.*, (2018). Microscopic visualization of glycogen deposits in cells and tissues is important for the study of normal glycogen metabolism as well as diagnosis of glycogen storage diseases. Lipids serve specific roles in the body, energy store, cell signals, and formation and maintenance of cell membrane. Triglycerides are deposited as fat in tissues and provide thermal insulation. In this study an effort was made to investigate the effect of *Tridax* leaf extract on the chick embryo blood vessels and collected data of essential biomolecules including the carbohydrates and lipids. *Tridax procumbens* (L.) is very commonly used as an herbal medicine in healing wounds (Mhaske and Gonjari; 2016; Pendharkar, 2021). Therefore, the histochemical effect of *Tridax* leaf extract is studied on the relative concentration of carbohydrate and lipid.

Materials and Methods

Freshly laid fertilized eggs (0 h stage) of *Gallus domesticus* (White Leghorn Strain) were obtained from poultry. Eggs were washed with distilled water and wiped with 70% ethanol and then incubated for 48 and 96 h in BOD incubator at 37.5 °C with a relative humidity of 70 –80%. The

identification of chick embryonic developmental stage was done (Hamburger and Hamilton, 1951). The chick embryos were treated as control (untreated embryo) and some of chick embryos were treated with 1 ppm, concentration of *Tridax* leaf extract. Plant extract treatment was given *in ovo* through air sac route at 46 h (HH stage 11-12) and incubated till 96 h (4 days incubation; HH stage 24) and transferred to the incubator set at 37.5°C. The treated and control eggs were manually rotated periodically. Chick embryo vitelline blood vessels network were used for glycogen and lipid estimation (Gado *et al.*, 2016). Histochemical studies were performed on fixed, paraffin-embedded tissues from early chick embryo vitelline blood vessels to demonstrate the glycogen and lipid residues in vascular tissue (vitelline membrane).

The estimation of glycogen content was performed by method of Kemp and Van Heijningen (1954) from 0.170 mg tissue. The optical density was taken with the help of spectrophotometer at 620 nm. The amount of glycogen in aliquot used was thus calculated by the formula:

$$\text{Glycogen (in mg) in aliquot} = \frac{100 \times U}{1.11 \times S}$$

Where U= O.D. of the unknown test solution;
S= O.D. of the 100 mg of glucose standard=3; 1.11= conversion factor of glucose to glycogen.

The lipid estimation was done using the chloroform-methanol method. The tissue of embryo isolated and used for lipid estimation. The tissue 0.200 mg was blotted then samples were dried in oven at 80 °C to remove excess water content and weighed and final dried tissue sample 0.170 mg was used for estimation. The dried samples were crushed with Na₂SO₄ (Anhydrous) separately by mortar and Pestel. The crushed tissue samples were mixed separately with mixture of chloroform and methanol (2:1, V/V) and filtered. An equal volume of 0.05 KCl was added to filtrate to remove non-lipid contaminants and release the bound acidic lipids. The KCl

washed supernatant was separated and residue was transferred to the beaker (pre-weighed) and allowed it to dry for 2 days in vacuum desiccator and completely dried residue was weighed.

The percentage of lipid was calculated as:

$$\% \text{ of lipid} = \text{wt. of lipid} / \text{wt. of tissue} \times 100$$

For the histochemistry the chick eggs were incubated at 37 °C in the laboratory up to 96 h (4 days). Glycogen histochemistry in the developing blood vessels were studied using alum hematoxylin. The tissue was fixed in Conroy's fluid. After fixation the tissues were transferred in absolute alcohol for dehydration then cleared in xylene and embedded in paraffin in usual manner and cut at 8-10 µm. The sections were deparaffinized and transferred through alcoholic grades up to tap water. Then stained in alum hematoxylin for 10-15 min, washed briefly in tap water and stained in Best's Carmine staining solution for 30 min. After staining slides were rinsed in Best's differentiator solution and washed in 90% alcohol briefly and placed in absolute alcohol. Then cleared in xylene and mounted in DPX (Forget and Doust, 1970; Postma *et al.*, 1978). Glycogen stains in magenta color.

For lipid histochemistry the control and treated tissues were fixed in Mercuric chloride and Potassium dichromate for three days. After fixation the tissues were rinsed in distilled water then dehydrated in alcohol grades, cleared in xylene, and embedded in wax in usual manner. The sections were cut at 8 µm. Then deparaffinized slides were transferred into absolute alcohol for 2 min and then transferred to absolute ethylene glycol and stained in Sudan Black solution for 30 min. The stained slides were differentiated in 85% ethylene glycol for 2-3 min, washed in distilled water and mounted in glycerin jelly. Fat stains blue-black in color (Elleder and Lojda, 1971).

Results and Discussion

In 48 h chick embryo (HH 12) 26 somite pairs are present and the anterior neuropore has closed. The developing head starts rotating to the left and

the primary optic vesicles become distinct. The heart is S-shaped and the forebrain is completely covered by the head-fold of the amnion until the next stage, the head is almost fully turned to the left, and the telencephalon is distinct and enlarged. At that point, the head-fold of the amnion completely covers the forebrain, midbrain, and the anterior portion of the hind brain (Hamburger and Hamilton, 1951). In control and treated embryos, Vasoturm (vessels of the vessels) and their capillaries are uniformly developed on right and left side of the embryo. The PBV (primary blood vessels) are normally developed with proper dilated manner and tapering further. The SBV (secondary blood vessels) and TBV (tertiary blood vessels) showed growth pattern like PBV (Fig. 1) (Mhaske and Gonjari, 2016). In 96 h chick embryo (HH stage-21), the entire body has been turned through 90 degree and the embryo lies with its left side on the yolk. The yolk stalk become elongated, allowing the embryo to become first straight in the mid-dorsal region and then convex dorsally. The progressive increase in the cranial, cervical, dorsal, and caudal flexures results in the bending of the embryo on itself so that its originally straight long axis becomes C-shaped, its head and tail lie close together. Optic cup shows the more developed lens. Endo-lymphatic duct arises from the auditory vesicle. Visceral arches have become very much thickened. Appendage buds increase rapidly in size and become elongated. The number of somites increases to 41 pairs. Allantois has also appeared. Omphalomesenteric artery and omphalomesenteric vein are also developed. In control and treated The PBV (primary blood vessels) are developed normally with proper dilated manner and tapering further. The SBV (secondary blood vessels) and TBV (tertiary blood vessels) show growth pattern like PBV (Fig. 2).

The microscopic observation of the wall of an artery shows that it consists of three layers. The innermost layer, the tunica intima (also called tunica interna), is made up of simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibers. The middle layer called tunica media primarily consists of

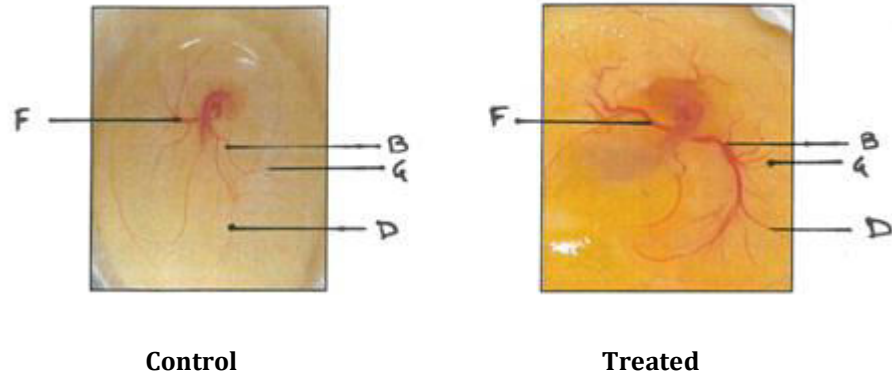


Fig. 1: 48 h chick embryo.

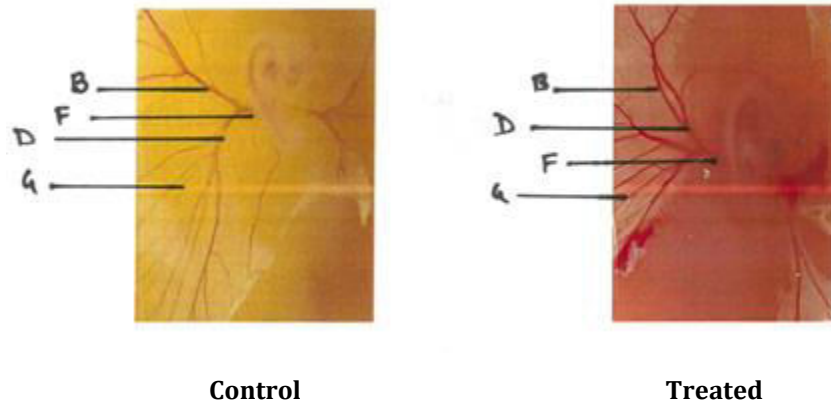


Fig. 2: 96 h chick embryo.

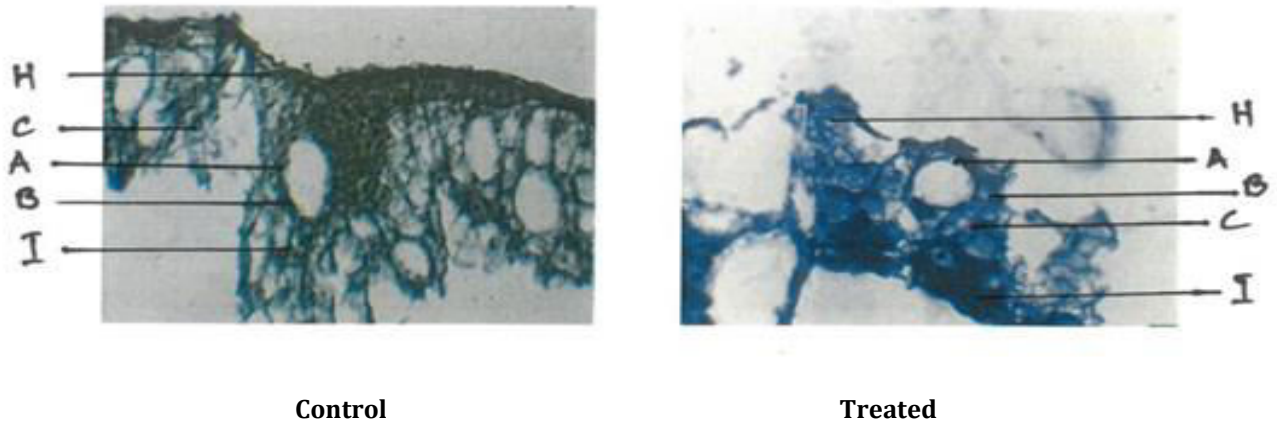
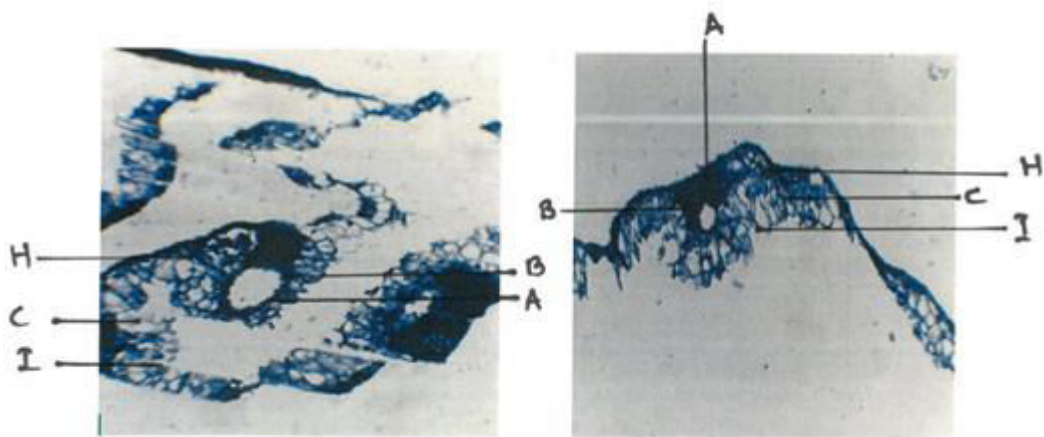
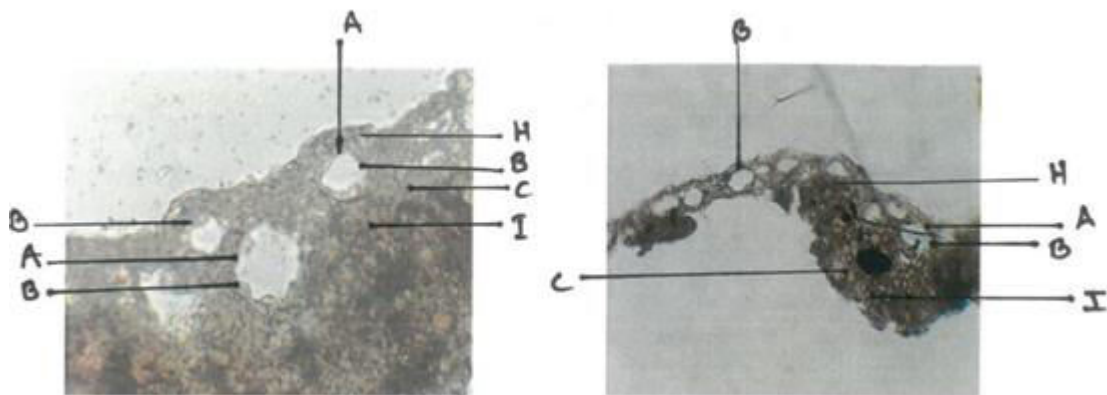


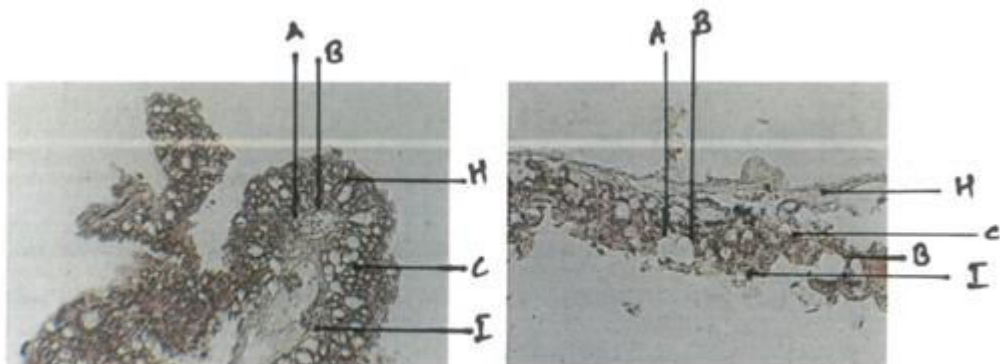
Fig. 3: Glycogen content in vitelline blood vessel of 48 h chick embryo.



Control **Treated**
Fig. 4: Glycogen content in vitelline blood vessel of 96 h chick embryo.



Control **Treated**
Fig. 5: Lipid content in vitelline blood vessel of 48 h chick embryo.



Control **Treated**
Fig. 6: Lipid content in vitelline blood vessel of 96 h chick embryo.

Abbreviations for Figs. 1-6: (A) Tunica externa; (B) Vitelline blood vessel; (C) Mesoderm; (D) Secondary blood vessel; (E) Endothelial cells; (F) Primary blood vessel; (G) tertiary blood vessel; (H) Ectoderm; (I) Endoderm

Table 1: Glycogen content of chick embryo

S. No.	Tissue	Incubation	Glycogen content
1	Control	48 h	0.003 mg/0.170 mg of tissue
2	<i>Tridax</i> extract	48 h	0.004 mg/0.170 mg of tissue
3	Control	96 h	0.006 mg/0.170 mg of tissue
4	<i>Tridax</i> extract	96 h	0.006mg/0.170 mg of tissue

Table 2: Lipid content of chick embryo

S. No.	Tissue	Incubation	Lipid content
1	Control	48 h	0.07 mg/0.170 mg of tissue
2	<i>Tridax</i> extract	48 h	0.07 mg/0.170 mg of tissue
3	Control	96 h	0.08 mg/0.170 mg of tissue
4	<i>Tridax</i> extract	96 h	0.08 mg/0.170 mg of tissue

smooth muscle and is usually the thickest layer. The outermost layer, which attaches the vessel to the surrounding tissue, is the tunica externa or tunica adventitia. This layer is connective tissue with varying amounts of elastic and collagenous fibers. Glycogen content in treated tissue is clearly visible magenta color in the wall of the blood vessel, there is no difference in the content of glycogen therefore it is very clear that plant extract has no effect on glycogen content. The middle layer of tunica media is thick and with smooth muscle layer, the inner layer is longitudinally oriented flattened cell linings of the lumen are also thick and show clear lumen (Figs. 3, 4). The glycogen content at 48 h is 0.003 mg in control and 0.004 mg in treated tissue (Table 1). There is no significant effect of plant extract on glycogen content.

In the tissues for lipid histochemistry, tunica externa of vitelline blood vessel is thick fibrous connective tissue layer, the middle layer of tunica media is also thickened with some smooth muscle layer to that of the tunica externa, the inner layer is of longitudinally oriented flattened cell lining of the lumen thickened. Overall thickness with normal lumen is observed (Figs. 5, 6). The estimated lipid content by chloroform-methanol method in control and plant extract treated tissue in 48 h incubated embryonic tissue is 0.07 mg and

in 96 h tissue it is 0.08 mg (Table 2). There is no significant effect of plant extract on lipid content.

Acknowledgements

Author is sincerely thankful to UGC, WRO Pune 7 for funding the minor research project (File No: 47-698/13(UGC-WRO). The author is thankful to the Principal Dr. R.G. Pardeshi, Fergusson College (Autonomous) Pune, for his constant support and help in the completion of this research project.

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