



Isolation, Characterization And Partial Purification Of Esterase From *Vigna Aconitifolia* Seeds

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Abstract:

Esterase plays a vital role in the biochemical mechanism during seed germination and associated with multiple aspects of plant physiology and developments. It is used as food additives to enhance the flavour, texture and taste of the foods, in making cheese, milk derivatives. This enzyme play role to activate prodrugs in vivo and generate effective anticancer agents in highly selected target site at the surface of tumor cells. Esterase was partially purified by a step wise purification methods like crude extract, ammonium sulphate fractionation, CM-Cellulose and Sephacryl G-200 chromatography from endosperm of. *Vigna aconitifolia*. Molecular weight of partially purified enzyme is 25 kDa approximately and determined by SDS-PAGE. The enzyme was fairly stable at 37 °C and pH 6.0. The specific activity and yield percentage of the enzyme were found to be 0.01µmoles/min/mg and 0.073 respectively. The Km and Vmax was 0.090 mM and 0.0111 µmoles / min.

Index Term: *Vigna aconitifolia* esterase, purification, yield

INTRODUCTION

Vigna aconitifolia is a draught resistant leguminous crop commonly known as moth bean, mat bean, turkish gram, moth gram, dew bean, dew gram. This crop belongs to the family Fabaceae, usually grown in arid and semiarid regions of India. Moth bean is mainly grown for food and forage. It has been cultivated in the United States, Australia, Thailand and others parts of Asia also [1]. India's most dehydrated state, Rajasthan, which contributes about 86 % of moth growing region [2]. 1.5 million hectares of terrain is used in India for moth bean manufacture. Whereas its existence in Somalia, Sudan and other tropical countries of Africa has been renowned. The prospective of increased production in this province in the future has been recommended.[4]. The seeds of *V. Aconitifolia* contains 0.20 % of L-dopa which is mainly used to treat Parkinson's disease. Mature unprocessed beans contain: water, energy, protein, fat, carbohydrate, several minerals like (Calcium, Magnesium, Phosphorus, Iron, Zinc), Vitamins like (Vit A, thiamine, riboflavin, niacin, Vit B6, folate and ascorbic acid). It also contains several amino acids like tryptophan, lysine, methionine, phenylalanine, valine, leucine, and isoleucine in edible portion along with several fatty acids which includes linoleic acid, palmitic acid, linolenic acid, oleic acid and stearic acid according to

(United State Department of Agriculture USDA, 2005) [3]. Sprouts of moth beans contain total soluble proteins, and nutritional and antioxidant properties. The highest albumin and globulin content [2]. Herbs can be used as an alternative remedy for different neurological disorders. Different bioactive compounds isolated from herbs are being successfully used for the treatment of neurological disorders [6]. Moth beans have several properties like antibacterial, antifungal, antiviral, anticancer and anti-inflammatory as it contains alkaloids, glycosides, essential oils, polyphenolic compounds and some other unusual substances [4]. Traditionally seed of *V. aconitifolia* was used in healing of paralysis, for weight reduction, rheumatism, cough, fever and liver ailments [5]

Esterase used as food additives to enhance the flavour, in making cheese, milk derivatives, texture and taste of the foods. Esters are also important for the aroma of fermented beverages like wine with ethyl acetate as the most common ester in wine due to its ready formation from ethanol and acetic acid. High reactivity of a primary alcohol and its low concentration gives specific fruity character to the wine [6]. In the present study the seed of *V. aconitifolia* was found as a promising source of esterase

MATERIALS AND METHODS

MATERIALS

(a) The dry seeds of *Vigna aconitifolia* were collected from D mart, Gopalan Mall, Mysore Road, Bangalore, Karnataka, India, during the month of June and August. With $KMNO_4$ solution seeds were surface sterilized and stored in airtight container for further use.

(b) α -naphthol, α -naphthyl acetate, acetic acid, acetone, ammonium persulfate, ammonium sulphate, acrylamide, bovine serum albumin, bromophenol blue, CM-sepharose, coomassie brilliant blue R-250, copper sulphate, diazoblu B, disodium hydrogen phosphate, fast blue RR, Folin Ciocalteu's Reagent, glycerol, methanol, N, N, methylene bisacrylamide, Sephacryl G-200, sodium dihydrogen phosphate, sodium hydroxide, sodium dodecyl sulphate, sodium chloride.tetramethylethylenediamine, tris base were purchased from SD fine, GE Health care, SRL Chemical Ltd and Thermos Fisher. Molecular marker (10-50 kDa) were purchased from Biocompare Lab.

METHODS

- (a) **Extraction of crude enzymes:** Soaked seeds (24 hrs) were taken and dehulled and endosperms used for extraction. 10 %, 20% and 30 % extract were obtained using chilled 30 mM phosphate buffer pH 7.0 using mortar and pestle. The extracts were centrifuged at 7,000 rpm for 15 mins at 4 °C. Pellets were discarded and supernatants were used for assay of esterase.
- (b) **Partial purification of enzymes by fractional precipitation method:** 10 % of extract were subjected to various fractional precipitation like change of pH, addition of chilled acetone and addition of ammonium sulphate.
- pH precipitation:** pH precipitation was carried out by adjusting the pH to 5.0 with 1 M acetic acid to crude extract and allowed to stand 30 mins. After centrifugation pellet was dissolved in 33 mM phosphate buffer pH 7 and then centrifuged to remove insoluble residues. The pH of the supernatant obtained was readjusted to pH 6 with 1M ammonium hydroxide. The residue and supernatant were collected in each case and assayed for esterase activity and protein.
 - Acetone precipitation:** Acetone precipitation was carried out by adding chilled acetone to the crude extract to obtained 0 - 40 % fraction. The solution was immediately centrifuged and the pellet was dissolved in minimum volume of 33 mM phosphate buffer pH 6.0. The supernatant was subjected to 40-70 % fractionation as described above and the pellet were dissolved in a minimum volume of 33 mM phosphate buffer pH 6.0 centrifuged to remove insoluble residues and assayed for esterase activity and protein.
 - Ammonium sulphate precipitation:** To the crude extract, finely powdered ammonium sulphate was added by constant stirring over a magnetic stirrer for 20 mins to obtained 0 – 30 % saturation. The solution was allowed to stand at 4 °C for 30 mins followed by centrifugation. The pellet obtained was dissolved in minimum volume of phosphate buffer. The supernatants obtained, were subjected to 30–60 % and 60–80 % saturation as described earlier. The pellets obtained from 40–60 % and 60–80 % saturation was dissolved in minimum volume phosphate buffer. The fractions were dialyzed against the 5 mM phosphate buffer pH 6.0 for 24 hrs with intermittent change of buffer. The supernatants were collected and centrifuged to remove the insoluble residue. The clear supernatant was used for the assay for esterase activity and protein.

(c) Partial purification of enzymes by chromatography method:

- (1) CM Cellulose Chromatography:** Ammonium sulphate fractionation (30 – 60 %) was carried out twice and pellets were pooled and dialysed against 3.3 mM phosphate buffer pH 6. The dialysed sample was loaded on to CM cellulose column. The CM cellulose was packed into column (2cm x 16 cm; 50 ml) under gravity and equilibrated with 20 mM phosphate buffer, pH 6. The flow rate was adjusted to 40 ml /hr. The bound proteins were eluted stepwise by an increase in ionic strength (0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M sodium chloride) and 5 ml fractions were collected. Assay of esterase was carried out as mentioned and proteins were estimated at 280 nm for each fraction. The fractions were assayed for esterase activity. Proteins were estimated at 280 nm.
- (2) Gel filtration chromatography:** The gel was equilibrated with 33 mM phosphate buffer pH 6.0 and packed into a column (1cm × 100 cm; 78ml) under gravity. The column was equilibrated with 33 mM phosphate buffer pH 6.0 with a flow rate of 7 ml/hr. 1 ml of the pooled CM cellulose fraction was loaded on to the column. 1 ml fractions were collected. The fractions were assayed for esterase activity. Proteins were estimated at 280 nm.

(d) Gel electrophoresis (Native PAGE and SDS)

- (1) Native PAGE:** A discontinuous gel system of 10 % separating gel and 5 % spacer gel was used. The sample (40 µl) was suitably diluted with bromophenol blue and 40 % glycerol. The sample was then loaded into wells of gel and subjected to electrophoresis at 4 °C applying a current of 150 mA. After the run, the gel was removed and incubated in 33 mM phosphate buffer pH 6 (esterase) for 30 mins.
- (2) SDS PAGE:** A discontinuous gel system of 12 % separating gel and 5 % spacer gel was used. 1 ml of treatment buffer contains partially purified protein from gel filtration fraction mix with equal volume of treatment buffer and heated in boiling water bath for 90 sec. The partially purified protein from gel filtration chromatography fractions were subjected to SDS PAGE. The marker protein (1-2 mg) was dissolved in 10 mM phosphate buffer pH 6.0 and partially purified proteins obtained from ion exchange and gel filtration chromatography were subjected to SDS PAGE. Marker proteins are obtained from Biocompare Lab with molecular range (10-50 kDa).

Staining of gels According to Bhavith *et al.*, [9] gel was stained for esterase activity with 50 ml of 33 mM phosphate buffer pH 6.0 containing 5 mg of fast-blue RR and 20 mg of 1-naphthyl acetate (dissolved in 1.0 ml of acetone) for 20 mins at room temperature.

The gel was stained for proteins using staining solution (0.01% Coomassie brilliant blue R-250 prepared in methanol, acetic acid and water (4:1:5) and destained in solution containing methanol, acetic acid and water (4:1:5).

(e) Kinetic Studies of enzyme

- (1) Effect of Time:** The optimization of time for enzyme activity was determined by incubating enzyme 0,3,6,9,12,15,20 and 25 minutes.
- (2) Effect of substrate:** K_m and V_{max} were determined for enzymes using different substrate concentration (0.0015mM – 0.015mM)
- (3) Effect on temperature:** Optimum temperature was determined by assaying enzyme at different temperature (7, 15, 22, 28, 30, 37, 45, 52, 60, 70 °C). Temperature stability was determined by pre-incubating enzyme at different temperature and assaying for each enzyme at room temperature.
- (4) Effect on pH:** The optimum pH was determined by assaying enzyme in different pH buffers. (50mM acetate buffer pH 4.0, 4.5, 5.0, 5.5, 33 mM phosphate buffer pH 6.0, 6.5, 7.0, 7.5, 50mM tris-HCl buffer pH 8.0, 8.5, 9.0) for esterase. pH stability was determined by pre-incubating enzyme at above mentioned pH for 20 min and carry out the assay for each enzyme

Assay of Esterase: The reaction mixture consists of 1.0 ml of suitably diluted extract and 5.0 ml of 0.3 mM α -naphthyl acetate (a stock of 30mM α -naphthyl acetate prepared in acetone and diluted 1:100 times in 30 mM phosphate buffer, pH 7). The reaction was carried out for 20 mins at room temperature. The reaction was stopped by the addition of 1.0 ml DBLS reagent (5 % sodium dodecyl sulfate and 1% diazoblu B mixed in the ratio 5:2). Absorbance was measured at 600 nm against a suitable blank [7].

Estimation of protein by Lowry's method: Protein estimation was carried out according to Lowry *et al.*, 1951 [8]. 1.0 ml of appropriately diluted supernatant and 5.0 ml of copper reagent were incubated for 10 mins at room temperature, 0.6 ml of Folin Ciocalteu's reagent added and allowed to stand for 20 min to develop the colour. Absorbance was measured at 660 nm against a blank.

RESULT AND DISCUSSION

- (a) The efficiency of extraction of enzymes from the seeds of *Vigna aconitifolia* with different percentage of buffers are shown in **Table 1**. Esterase extracted with 20 % phosphate buffer pH 6 shows maximum activity.
- (b) **Table 2** shows different fractional precipitation methods of crude enzymes. Maximum total activity of esterase (2.138 μ moles/ min/g) with 30-60 % ammonium sulphate precipitation was chosen for further purification.
- (c) **Partial purification of esterase:** Ammonium sulphate fraction (30-60 %) was subjected to CM cellulose chromatography and resolved into peaks; (**Figure 1**) and was further pooled purified by gel filtration chromatography using sephacryl G-200. One broad major peak was obtained from 33th fraction (**Figure 2**).
- (d) **Native PAGE:** The crude, ammonium sulphate fraction and ion-exchange chromatography fractions were subjected to native PAGE. Proteins and activity band patterns obtained shown in **Figure 3** which shows one band for esterase. SDS PAGE pattern for standard protein and partially purified esterase are shown in **Figure 4**. Molecular weight for partially purified esterase was approximately 25kDa obtained from ammonium sulphate fraction precipitation. One faint band was obtained ion exchange chromatography which is approximately 25 kDa.
- (e) **Determination of assay conditions** The assay condition for esterase enzyme are as follows: Optimum incubation time was 20 mins. The K_m and V_{max} was 0.090 mM and 0.0111 μ moles/ min. The optimum pH was 6.0 and stability range from pH 5.0 to 7.0. The optimum temperature was 37 °C. and the enzyme was stable upto 37°C

Partially purified esterase showing total activity, protein, specific activity, yield percentage and fold purification at each stage is shown in **Table 3**

Molecular weight of esterase isoenzymes obtained during the germination of various source are 28, 25, 22 kDa from *Sesbania grandiflora* seeds. Two isozymes were isolated from *Jatropha curcas* seeds with molecular weight 21.6-23.5 and 30.2kDa [10]. Esterase from *Cesalpinia mimosidae* seed was 20kDa [9]. The above results suggest that esterases are of low molecular weight ranges from 20- 30 kDa. The esterase from *V. fischeri* showed molecular mass of 37 KDa [11] Molecular weight of partially purified enzyme was found to be approximately 29 kDa from the germinated seeds of *Leucaena leucocephala* [14]. Due to versatile uses esterase is gaining research interest. Esterase obtained from *Cynara Cardunculus L.* and *Ficus Carica L.* is important to release odorous compounds in cheese making [12]. Carboxylesterase is required for plant growth and development and able to detoxify by interacting with three major classes of agrochemicals organophosphates and pyrethroids[10].The global market for industrial enzymes is constantly growing with a rate of 5-10 % per year. Esterase has so many industrial uses like in cosmetics, detergent composition, synthesis of carbohydrate derivatives and food additives [13].

CONCLUSION: *Vigna aconitifolia* is found to be a good source of esterase and this study promotes to meet never ending demands.

Table 1 Extraction of enzyme with different percentage of phosphate buffer pH 6

Percentage of Phosphate buffer	Activity (μ moles/ min/g)	Protein (mg)
10%	2.681	48.112
20%	2.70	54

Table 2 Fractional precipitation methods

Purification methods		Volume (ml)	Total activity (μ moles/min)	Total protein (mg)	Specific Activiy (IU/mg protein)
Crude		40	18.24	627.2	0.029
pH precipitation	Pellet1	10	1.31	112	0.0117
	Supernatan t	37	5.589	94.72	0.059
Acetone precipitaton	Pellet 1	5	0.556	21.6	0.0257
	Pellet 2	10	0.143	11.2	0.0013
Ammonium sulphate precipitation	0-30%	5	0.014	6.4	0.0022
	30-60%	15	2.138	45.6	0.0468
	60-80%	10	0.641	12	0.0534

Table 3. Purification Table of esterase

Purification step	Vol (ml)	Total Activity (μ moles/min s)	Total Protein (mg)	Specific Activity (μ moles/mi ns/mg)	Fold Purificati on	% Yield
Crude	182	82.992	221.312	0.375	1	100
Ammonium sulphate precipitation	12.5	4.346	164	0.027	0.072	5.237
CM-sepharose chromatogra phy	5	0,129	10.75	0.012	0.032	0.156
Sephacryl G-200 chromatogra phy	1	0.061	5.6	0.01	0.026	0.073

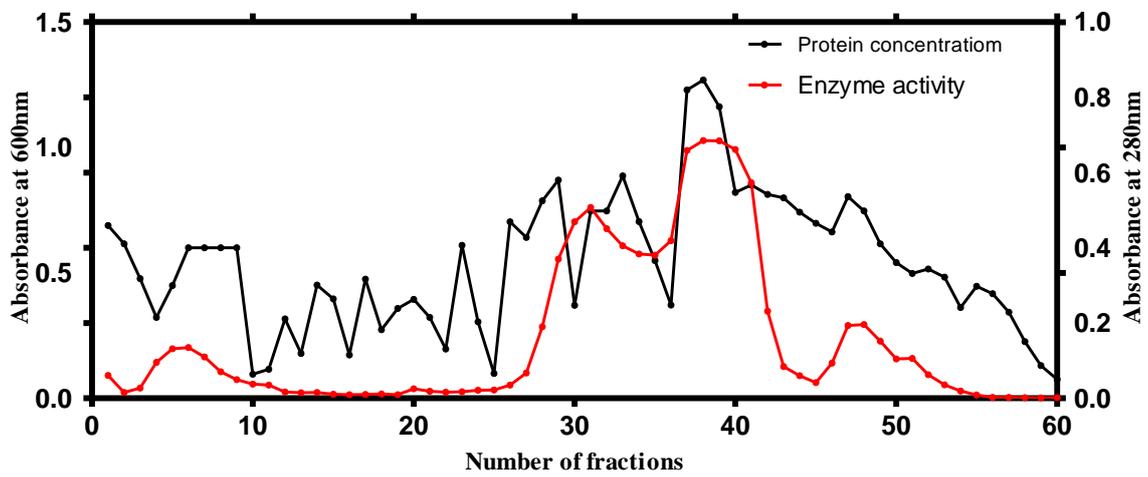


Figure1 Step wise elution profile of esterase from seeds of *Vigna aconitifolia* on CM Cellulose matrix

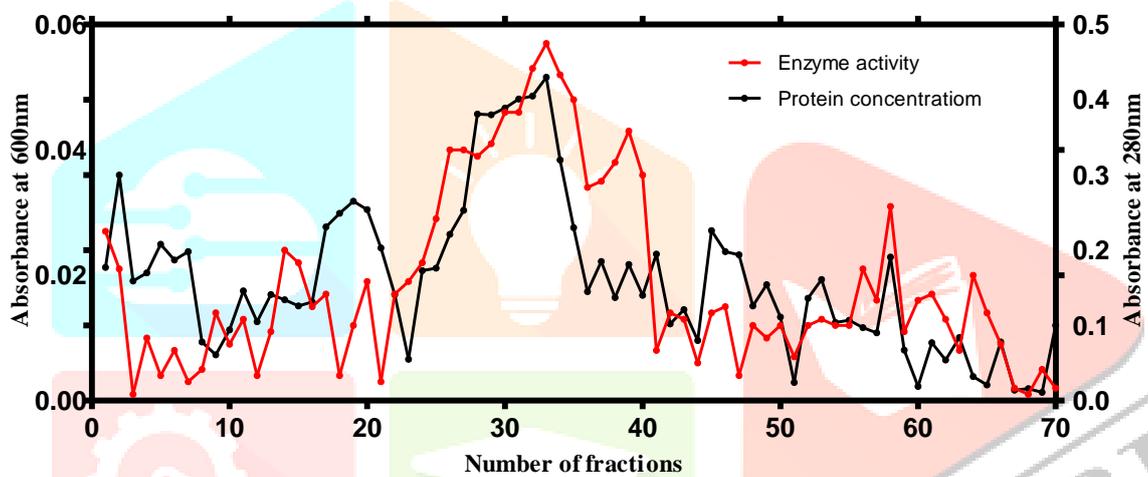
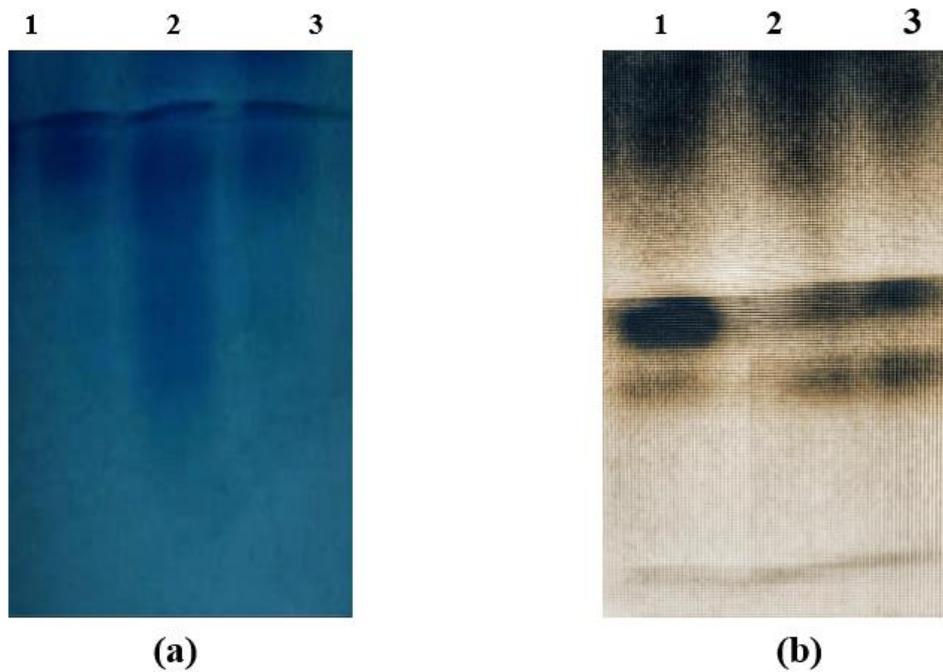


Figure 2: Elution profile of esterase from the seeds of *Vigna aconitifolia* on Sephacryl G-200 matrix



Lane 1 Crude. Lane 2 ammonium precipitation, Lane 3: Ion exchange chromatography

Figure 3 Native PAGE of (a) protein and (b) Activity of esterase from the seeds of *Vigna aconitifolia*

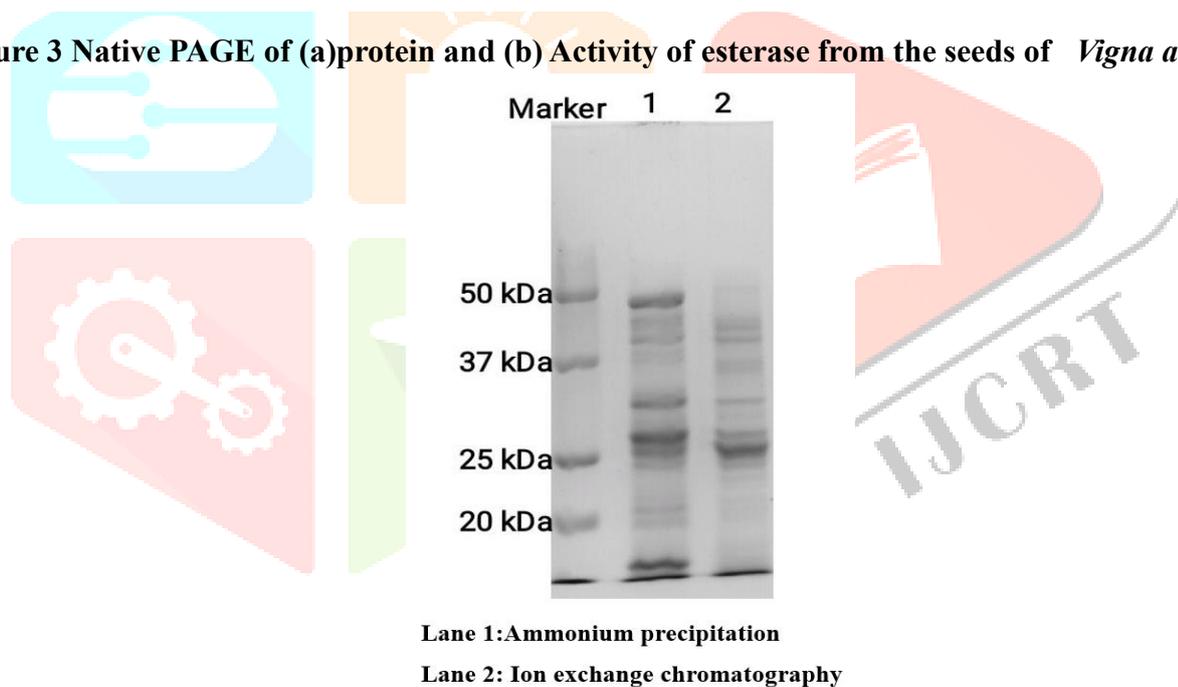


Figure 4 SDS- PAGE from seeds of *Vigna aconitifolia*

ACKNOWLEDGEMENT: The authors thank to Department of Biochemistry, Bangalore University, Jnanabharathi Campus, Bangalore 560 056, India for providing the facilities needed to perform this study.

CONFLICT OF INTEREST: The authors declare that we do not have a conflict of interest.

ETHICAL CLEARANCE: The research work does not involve any animals

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