



ANTIPLATELET AGGREGATION ASSAY OF HOMOGENATE OF *EUTYPHOEUS GAMMIEI*- A NATIVE EARTHWORM OF TRIPURA, NORTH-EAST, INDIA

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ABSTRACT

For several years earthworms have been widely used in china, Japan, Indonesia, and the Far East to treat various chronic diseases. Intensive exploration has been made to reveal the use of earthworm as anti microbial anti inflammatory, anti pyretic and anti-cancer agents. Earthworm contains many compounds with potential medicinal properties and have been administered to treat inflammatory, hematological oxidative & nerve disease. It has been reported that medicinal activities of earthworm may vary depending on the species & living environment of that organism Cellular metabolism plays a vital role in the homeostasis of the bodyof any living organism. This metabolism is disturbed in conditions like thrombus that inhibits the blood supply to the body tissues leading to ischemia and finally necrosis of the cells. Hence, there is an urgent need for research in the field of antiplatelet and thrombolytic agents in order to prevent and cure thrombus thatleads to pathological conditions like Myocardial Infarction (MI), Hemiplegia and the like. It is essential to search for mineral medicines of similar nature which aid in a speedy recovery and are considered to be cost effective with a longer shelf life. The extract of *Eutyphoeusgammiei* (*E.E.G*) is used as a drug.As found in the literature, it is also used in cardiac ailments predominantly Angina and Mi for which thrombus is the reason behind the pathological condition. In this study, the extract of *Eutyphoeusgammiei* was evaluated for its *in vitro* antiplatelet aggregation and thrombolytic activity which proved effective at the dose of 65µl and 100µl respectively. Therefore, it can be concluded that *Eutyphoeusgammiei* (*E.E.G*) extract is an effective drug in the treatment of Cardiovascular diseases and Cerebrovascular accidents.

KEYWORDS: *Eutyphoeusgammiei* Anti-platelet drug, Anti platelet aggregation, whole blood aggregation



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INTRODUCTION

From time immemorial, earthworm has been used as a therapeutic agent for curing different ailments. Earthworm has rather been widely used in China, Far East as a drug materials for treatment of different ailment for several thousand years. Earthworms contain many potential compounds for the treatment of inflammation, haematological, oxidative and nerve diseases. Among many properties, earthworm also been reported to exhibit antimicrobial, antiviral, anticancer and fibrinolytic activities Mihira *et al.*, had reported *Lumbricus rubellus* from Japan to be potentially very useful in treating thrombosis. In this ultra-modern world, we face major threats against many stress-related diseases. Thrombus is a pathological condition that plays a vital role in causing many diseases like Stroke, Deep vein thrombosis and Myocardial infarction. A major clinical manifestation of thrombus is stroke. Stroke or Cerebro Vascular Accident (CVA) Causes 2,00,000 deaths each year and is a major cause of disability. The most common forms of Cerebro Vascular Accident (CVC) are Cerebral thrombosis (40% of cases), Cerebral embolism (30% of cases) and Cerebral hemorrhage (20% of cases). Long before the inventions of various modern techniques and drugs, the Siddhars with their spiritual insights introduced various traditional medicines to prevent Stroke. It has been reported that *Cheenalings Chendhuram* can reduce platelet aggregation *in vitro*¹. From time immemorial earthworms have been used as a therapeutic agent. Recently earthworm protein and its coelomic fluid were reported to have cytolytic, agglutinating, proteolytic, haemolytic, mitogenic, anti-pyretic, tumorstatic and antibacterial activities²⁻⁴. Vohora and Khan⁵ found earthworms to have curative effect on wounds, chronic folds, piles and sore throat. Earthworm's anti-pyretic properties were reportedly tried in China and Japan in reducing fever. Anti-pyretic components were found in the earthworms *Lumbricus spp* and *Perichaeris spp* by Hori *et al*⁶. Bhatnagar and Palta⁷ have reported that earthworms when ingested into our body system increase body heat and are of value in curing neural disorders, bronchitis and tuberculosis including rheumatism. Mihara *et al*⁸ have reported *Lumbricus rubellus* to be potentially very useful in treating thrombosis. The orally administered earthworm powder was found capable of digesting intravascular fibrin clots. If glycoprotein (G-90) from earthworm tissue and the proteolytic enzymes such as PI and PII were administered, Popoviet *al*⁹ reported the presence of

anticoagulant and fibrinolytic activity in the blood of the dog with malignant tumors. Northeast India which is located in one of the mega biodiversity regions of the world is a source of diversified flora and fauna. The earthworm endemic to this region may be an excellent source of biotic compounds of medicinal values. *Eutyphoeus gammieii*, an earthworm species is endemic to North-East, India¹⁰. However the species has not yet been molecularly characterized. The medicinal use of Earthworm has been practiced for many centuries to cure various diseases in China and other parts of far East¹¹. The extract of *Eutyphoeus gammieii* is one among them which is capable of thrombolytic activity and anti platelet aggregation activity. Although there are many Antiplatelet aggregation and Thrombolytic medicines in Siddha, no proper scientific evaluation is made. No attempt has been made to establish the extract of *Eutyphoeus gammieii* as a good anti-platelet aggregation thrombolytic drug in siddha system. These beneficial effects result in the improvement of blood fluidity. However, as the siddha drug significantly enhances fibrinolytic activity, it is theoretically possible that its over-activity could cause the platelets to aggregate through the release of fibrinogen degradation products because it has been reported that excessive fibrinolysis resulted in the release of FDP. These findings clearly showed that siddha drug has anticoagulant properties. Its medicinal uses include treatment for Stroke, Deep vein thrombosis and Myocardial infarction. The interaction between the platelets and the walls of blood vessel walls are important in the development of thrombosis and cardiovascular diseases such as Myocardial infarction, Stroke and Atherosclerosis. Among the family of Platelet Activating Factors (PAF), Arachidonic acid and ADP are the three important platelet stimulants which induce platelet aggregation via different mechanism. Platelet plays a key role in the physiological hemostatic process. The pathologic thrombosis extract of *Eutyphoeus gammieii* possesses anti-platelet activity that plays an important role in preventing thrombotic events thereby decreasing the risk of cardiovascular diseases. Platelets are involved in the pathogenesis and progression of cardiovascular diseases. These beneficial effects result in the return of normal blood flow. Inhibitory effect of drugs on platelet aggregation has been generally estimated by measuring the change in light transmission rate of the Platelet Rich Plasma (PRP) (Born & Cross, 1963)¹². However, this differs from the case with whole blood. PRP contains only platelets and not erythrocytes and leukocytes both of

which might affect platelet aggregation by releasing substance or other interaction. Lumbrokinase extract derived from the earthworm evinces its benefit in lowering the viscosity of whole blood and in reducing platelets aggregation¹³.

METHODOLOGY

Collection of Specimen & Identification

Around fifty adult earthworms *E. gammiei* were identified by digging, hand-sorting, and wet sieving method (Edwards and Lofty, 1977; Reynolds,

1977)¹⁴⁻¹⁵. They were collected with the help of a spade from Agartala, Tripura in the early morning. The site was demarcated using GPS (Latitude: N =23.82914/23.8213 and Longitude: E=091.29485/091.29484). The sample was first identified by Prof. P.S.Choudhuri, Earthworm Research Laboratory, Department of Zoology, Tripura University. The sample was sent to ZSI, Kolkata for identification. The sample was identified by ZSI, Kolkata with the registration number –An 5649/1



Figure 1
Eutyphoeus gammiei

Preparation of tissue homogenate

The collected specimens of Earthworm – *Eutyphoeus gammiei*- were washed in running water and then cleaned with wet blotting paper for 18-20 hrs to remove their gut. The worms were then washed in distilled water. Twenty percent of the gut cleared earthworm homogenate was prepared¹⁶ in 0.2M PBS, pH-7.2. The Homogenate was filtered and sterilized before use.

Estimation of Protein concentration

Protein concentration was determined by the Lowry's method. The Lowry method is based on the reaction of Cu^+ , produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction)¹⁷

Anti Platelet Aggregation

Platelets rich plasma (PRP) was prepared by centrifugation (250g for 5 min) of blood collected from normal aspirin-free blood donors. 1.5 ml of acid citrate dextrose was used as an anticoagulant for every 8.5 ml of blood. PRP was taken into siliconized glass. Platelet poor plasma (PPP) was prepared by centrifugation at 4500 g for 5 min and was kept as reference. The cuvettes were incubated at 37⁰ C for 5 min. The aggregation was initiated by adding 20 μl of ADP (10 μM) to 1 ml of PRP. The aggregation was recorded for 5 min at 600 nm. The effect of different concentrations (20 μl , 40 μl , 65 μl , 100 μl) of *Eutyphoeus gammiei* homogenate was studied by incubation with PRP at 37⁰ C for 5 min before the addition of ADP. Commercial heparin (20 $\mu\text{g}/\text{ml}$) was used as a standard. The maximum aggregation was recorded. The aggregation is expressed as % inhibition (X) which is calculated by using the equation¹⁸:

$$X(\%) = A - B/A \times 100$$

Where A = maximal aggregation of the control, and B = maximal aggregation of drug treated PRP.

STATISTICAL ANALYSIS

The data of In vitro anti-platelet aggregation activity of *Eutyphoeus gammiei* was analyzed statistically using One Way ANOVA followed by Student's (paired) "t" test by computer software programme. The data were presented as mean \pm standard deviation (SD). Probability value (p) of less than 0.05 was considered statically significant.

RESULT AND DISCUSSION

Platelets play a pivotal role in health and diseases, given their central involvement in homeostasis and thrombosis. Recently several natural anti-platelet agents from natural products including polyphenols and flavonoids have been reported. Plant preparations containing polyphenols/flavonoids have been used for centuries as herbal remedies for a variety of diseases and found to have an impact on diabetes and obesity related disorders. Thrombosis plays an important role in the pathogenesis of acute coronary syndromes, and vessel wall injury leads to the adherence of platelets and subsequent platelet activation. Platelet aggregation is absolutely essential to the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction. Inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. Homogenate of *Eutyphoeus gammiei* may have an anti-platelet function by elevating the cyclic adenosine monophosphate (cAMP) level, and then by decreasing the $[Ca^{2+}]$, an essential factor for platelet aggregation. Intravascular thrombosis is one of the generators of a variety of cardiovascular

disease and platelet aggregation is believed to play a crucial role in atherothrombotic processes. It is reported that *Cheenalunga Chendhuram* have anti-platelet aggregation activity. In vitro, *Cheenalunga Chendhuram* (40–200 μ M) inhibits platelet aggregation in a dose-dependent manner stimulated by an agonist ADP, in human platelet rich plasma. Further investigation reveals that those effects are due to the inhibition of phospholipase C activity, leading to reduced phosphoinositide breakdown, followed by the inhibition of thromboxane A₂ formation, and then inhibition of $[Ca^{2+}]$ mobilization of platelet aggregation stimulated by agonists. Tissue factor (TF), also called platelet tissue factor, is necessary for the initiation of thrombin formation from the zymogen prothrombin. Besides the above mechanism, the antiplatelet activity of *Cheenalunga Chendhuram* is related to the inhibition of the release of platelet-derived TF by stimulating the synthesis and releases of cGRP (cyclic g\Guanosine Releasing Peptide). Taken together, all these results suggest that *Cheenalunga Chendhuram* has an effective anti-platelet effect both in vivo and in vitro, and be a potential therapeutic agent for arterial thrombosis¹⁹. In order to examine the antiplatelet aggregation property of *Eutyphoeus gammiei*, antiplatelet aggregation activities of *Eutyphoeus gammiei* homogenate against ADP-induced platelet aggregation were measured depending upon the dose. The doses fixed for the antiplatelet aggregation activities were 20 μ l (26.4 μ g), 40 μ l (52.80 μ g), 65 μ l (85.80 μ g), 100 μ l (132 μ g). It was clearly observed that *Eutyphoeus gammiei* homogenate inhibited platelet aggregation in a dose dependent manner (Table 1, Fig.2) The Data obtained demonstrate substantial anti-platelet aggregation activities of *Eutyphoeus gammiei* homogenate.

Table 1
ADP induced Platelet aggregation inhibition (%) at different doses of
***Eutyphoeus gammiei* homogenate**

Figure No.	Contents	CONTROL TUBE (Normal Saline) : %	Test Tube (Homogenate of <i>Eutyphoeusgammiei</i>): %	Inhibition of platelet aggregation (%)
1	450 µl PRP + 20µl sample (C/T)+30ul ADP (6 µM)	88.87±0.61	69.97±0.55	20.96±0.45
2	430 µl PRP + 40 µl sample (C/T)+30 µl ADP (6 µM)	86.7±0.53	55.33±1.04	35.67±1.04
3	305 µl PRP + 65 µl sample (C/T)+ 30 µl ADP (6 µM)	89.53±0.61	19.9±0.36	76.97±0.45
4	370 µl PRP + 100 µl sample (C/T) + 30 µl ADP (6 µM)	84.97±0.45	14.97±0.45	81.87±0.61

± SD , p< 0.05

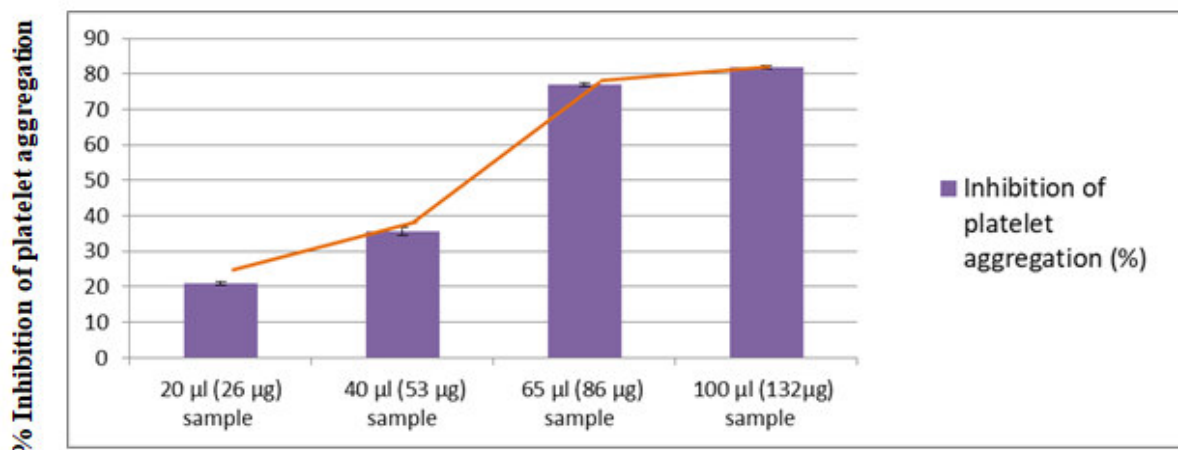


Figure 2
Platelet aggregation inhibition (%) by Homogenate of *Eutyphoeus gammiei*.

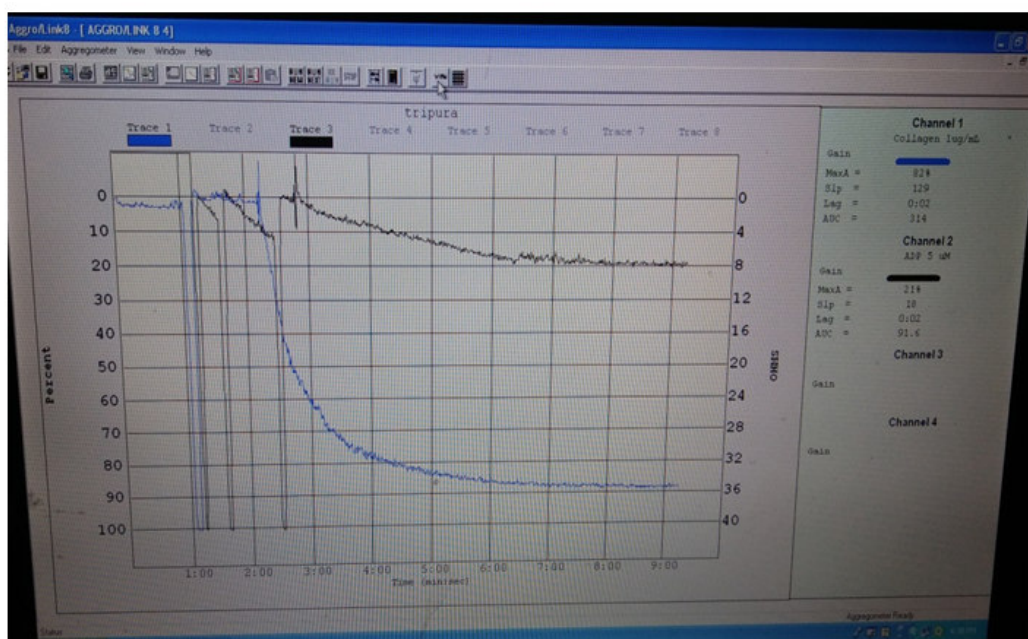


Figure 3
Showing reduction of aggregation to 20% in presence of test material. The curve moved down till 89% when only buffer was taken.

Platelets play a crucial role in thrombosis. Platelet activation in atherosclerotic arteries and is central to the development of Arterial thrombosis. *E. gammieii* homogenate has shown significant dose dependent inhibitory effect on ADP-induced human platelets aggregation (Fig. 2 and 3). It has already been reported by Jiet *al.*, 2010¹⁶ that Lumbrokinase has anti-platelet aggregation activity. According to them, the mechanism involved increased cellular AMP level and inhibition of Ca transport. Similar mechanism may also be involved in this case.

CONCLUSION

In conclusion, many Cardiovascular diseases can be attributed to excessive platelet aggregation. It appears that *Eutyphoeusgammiei* can inhibit platelet aggregation *in vitro*; The present study ensures the anti-platelet aggregation of *Eutyphoeusgammiei* which is beneficial for patients with cardiovascular disorder. However, this drug should be used with caution by patients with bleeding or other haematological disorders as it may increase the risk of bleeding and might lead to other complications.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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