ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Antioxidant and Anti-Inflammatory Properties of Autolysed Extract of the Indian Earthworm *Pheretima posthuma* after Preliminary Purification - An *In Vitro* Study

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ABSTRACT

The current study is aimed to investigate and evaluate the therapeutic potential of the dialysed protein extract of the Indian earthworm *Pheretima posthuma* for antioxidant and anti-inflammatory activities. The antioxidant activity was assessed by calculating Total Phenolic Content, Reducing power and Nitric Oxide Radical Inhibition capacity of the prepared autolysed earthworm extract. The anti-inflammatory activity of the prepared earthworm extract was determined by estimating the percentage of RBC membrane stabilization using Diclofenac Sodium, a non-steroidal anti-Inflammatory drug (NSAID) as standard for comparison. We have reported the total phenolic content as 8.80 µg GAE/mg of protein present in the extract, 37.90% of nitric oxide scavenging ability and considerable reducing activity of earthworm extract although less in comparison to the reference standard of Ascorbic acid. A high percentage (58.6%) of RBC membrane stabilization was noticed while incubating the extract with human RBC suggesting its strong anti-inflammatory activity *in vitro*. Further fractionation and purification of potent therapeutic molecules from the crude extract might lead to the exploration of novel drug leads of natural origin from the earthworms.

Keywords: Earthworm extracts, Total phenolic content, Gallic acid, Antioxidant, Nitric oxide scavenging, Anti-Inflammatory, RBC membrane stabilization.

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ISSN: 0975-8585

INTRODUCTION

The oxidative stress is a major concern in present scenario as prime cause of various diseases and disorders due to the production of reactive oxygen species (ROS) viz., Hydrogen peroxide (H_2O_2), Nitric Oxide, hydroxyl radical (OH`) and others that alter normal physiology and homeostatic balance. To remediate the harmful effects of oxidative radicals produced or consumed in biological systems, artificial/synthetic antioxidants are available to minimize oxidative stress. In addition to these synthetic antioxidants, there are numerous biological sources of antioxidant compounds from plants, microbes and animals [1-5]. Many investigations have shown animals, in addition to plants and microbes, as sources of diverse biomolecules exhibiting antioxidant property [6-12] Several studies have confirmed that protein hydrolysates possess anti-oxidant activity and that their capability to inhibit deleterious changes during lipid oxidation also depends on the presence of certain amino acid residues in the peptides like tyrosine, histidine, methionine and tryptophan. These amino acids are also capable of collecting pro-oxidant metal ions [9].

There are several methods of assessing the anti-oxidant properties of hydrolyzates viz., measurements of peroxide value, 2-thiobarbituric acid reactive substances (TBARS), anti-radical activity against the DPPH and hydroxyl radicals, superoxide scavenging activity , and ferric reducing/antioxidant power (FRAP). Results of experiments indicated that the anti-oxidant properties of the hydrolysates are dependent on the enzymes employed in hydrolysis and the amino acid composition of peptide fragments particularly the presence of cysteine, methionine, lysine and Valine [13]. The antioxidant effect of these amino acid residues was reported and is an established fact [14].

Inflammation is an essential part of defense system associated with vascular tissue often noticed with increased fluid accumulation to a local tissue along with increased temperature and redness. Inflammation is associated with a number of biological mediators such as histamine, 5-hydroxy tryptamine (serotonin), cytokines, bradykinins and numerous plasma proteins along with immune mediators such as mast cells, dendritic cells and antibodies [15]. Depending on the severity of inflammation, acute or chronic, anti-inflammatory agents such as NSAIDs and steroidal ones are administered. Synthetic anti-inflammatory agents are often associated with numerous side effects viz., gastric bleeding, nausea, liver and kidney complications [16]. Hence there is an imperative need for finding newer agents especially from biological origin to avoid/minimize side effects.

The diverse therapeutic potentials of earthworm proteases such as fibrinolytic activity, antiviral activity and anti-tumor activity have been reported in several investigations in the last few decades [9]. Anti-oxidant and anti-inflammatory properties of the earth worm pastes have also been reported [17-20] but to the best of our knowledge, no reports are available on the identification of individual protein fractions with respect to these functions. In the current study we have investigated earthworm proteases: a group of serine proteases existing as isoforms in the molecular weight range of 15-32 kDa and exhibiting potent antioxidant and anti-inflammatory activity confirmed by *in vitro* analysis. An attempt for ultra-purification of the

potential protein fractions is underway to possibly identify and establish the molecular association between the individual protein fractions and their medicinal potentialities.

MATERIALS AND METHODS

Preparation of Earthworm extract

Fully grown and mature Indian Earthworm species *Pheretima Posthuma* approximately 1kg collected from the local vermicomposting center were further grown for one week in soil with excess humus and repeated water spray under laboratory conditions. After one week, healthy and mature earthworms were subjected to autolysis after repeated washes with sterile distilled water. Analysis of cleaning earthworms was performed for 3h at 60^0C in 20mM phosphate buffer pH 7.5 with 0.02% Sodium Azide as bacteriostatic. Further to complete autolysis, earthworms were subjected to 15^0C for one week in refrigerator. After one week of autolysis, earthworms were subjected to successive high speed centrifugation at 16000 rpm for 30 min. at 4^0C. Clear supernatant was transferred into sterile tubes under aseptic conditions. Supernatant of 500 ml volume was filtered several times with Whatman filter paper to remove tissue debris, if any and then subjected to preliminary purification steps. The total protein content of crude extract of 500 ml was precipitated with ammonium sulfate and 65% salt employed to recover 100% of protein [21]. Precipitated protein part was separated from the supernatant by high speed centrifugation at 20,000 RPM for 30 min at 4^0C. The precipitate was suspended in 25ml of 20mM phosphate buffer pH 7.5 and subjected to dialysis. Dialysis was performed to remove salt from crude protein by using dialyzing membrane (HiMedia) of Molecular Weight Cut Off (MWCO) of 12 kDa. Dialysis was done against 20mM phosphate buffer pH 7.5 for 8 hours with gentle stirring at 4 ^{0}C. The buffer was replaced with fresh ones at an interval of 2h during dialysis. After the dialysis crude protein sample was transferred into sterile eppendorff tubes and was used for anti-oxidation activity tests. The protein content of the crude extract was quantified [22] by Lowry's method.

Methodologies for Evaluation of Anti-Oxidant Activity

The antioxidant activity of the purified protein isolate was evaluated by performing three assays one each for total phenolic content, reducing power and nitric oxide radical scavenging activity.

Total Phenolic Content

Total natural phenolic content of the earthworm isolate was determined using Folin-Ciocalteau assay [23] with minor modifications. Test tubes containing 500 µl of standard solutions of Gallic acid (50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 µg/ml) were prepared, 500 µl of 10% Folin-Ciocalteau's phenol reagent (in DDW) was added into each test tube and mixed. After 20 minutes, 350 µl of 1M Na_2CO_3 solution was added into the mixture. After incubation for 20 minutes at room temperature, the absorbance was determined at 750 nm against the prepared blank containing 500 µl of Double Distilled Water and 500 µl of 10% FC reagent and

350 µl of 1M Na_2CO_3 solution). Then 50 µl of the isolated protein has been tested following the same procedure for determining the phenolic content and expressed as µg Gallic acid equivalents (GAE)/mg of protein sample.

Reducing Power

Reducing the power of the purified protein isolate was determined according to the method of [24]. Different concentrations of the protein sample (0.15, 0.3, 0.75, 1.5 and 3.0 mg/ml) prepared in 1 ml of phosphate buffer were added to 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% Potassium ferrocyanide. The mixture was incubated at 50^0 C for 20 minutes. About 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm and compared with the standard of ascorbic acid.

Nitric Oxide Radical Inhibition

Nitric Oxide radical inhibition is estimated by the use of Griess Illosvoy reaction [25]. Nitric oxide is generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes for oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate–buffered saline (PBS) was mixed with 3.0 ml of different concentrations (25, 50,100, 200 and 400 µg /ml) of the ascorbic acid (standard) and incubated at 25 ^{0}C for 150 minutes. The samples were reacted with Griess reagent which is 1 part of 0.1% naphthyl ethylenediamine dihydrochloride in distilled water plus 1 part 1% sulfanilamide (or sulfanilic acid) in 5% concentrated H_3PO_4.

The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. The same experiment was performed with protein samples of different concentrations (0.3, 0.6, 1.5, 3.0 and 6.0 mg/ml) prepared in phosphate buffer solution.

The percentage scavenging of nitric oxide of the protein isolates and standard compound (Ascorbic acid) is calculated using the following formula:

NO Scavenged (%) = (Abs. control - Abs. test)/Abs. Control × 100

Where Abs. control is the absorbance of the control reaction and Abs. Test is the absorbance in the presence of the sample under study.

Determination of Anti-inflammatory Activity (Membrane Stabilizing Activity)

Preparation of Human Red blood Cells (HRBC) Suspension

Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid and 0.42 % sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min and packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

Hypotonicity Induced Hemolysis

The methodology used involves estimation of stabilization of human red blood cell membrane through measurement of the haemoglobin released by hypotonicity induced membrane lysis of RBC. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline [0.36 %], 0.5 ml HRBC suspension [10 % V/V] with 0.5 ml of earth worm extract (in the case of test sample) (EE) and standard drug Diclofenac Sodium (in the case of control) of various concentrations (50, 100, 250, 500, 1000, 2000 µg/ml) incubated at 37^{o}C for 30 min and centrifuged respectively. The hemoglobin content in the suspension of the test and the control was estimated using spectrophotometer at 560 nm.

The percentage of hemolysis of HRBC membrane was calculated as follows:

% of Hemolysis = (OD of Test Sample / OD of Control) × 100

The percentage of HRBC membrane stabilization was calculated using the formula

% of Membrane Stabilization = 100 - (OD of Test sample / OD of Control × 100)

RESULTS

The earthworm extract was prepared after complete autolysis and protein content was precipitated with 65% (W/V) $(NH_4)_2SO_4$. Later it was subjected to Dialysis for about 12 hours and the dialyzed extract was analyzed for protein concentration by Lowry's method [22]. The dialyzed earthworm extract (EE) with a protein concentration of about 30 mg/ml was used for the study of antioxidant and anti-inflammatory activities. Figures 1 to 4, drawn using Sigmaplot 10, represent the results of the various tests performed to ascertain the anti-oxidant and anti-inflammatory potentials of the extract.

Total Phenolic Content

Standard graph was plotted with varying Gallic Acid conc. as specified in protocol. Total phenolic content of earthworm extract was evaluated and interpolated from standard graph (Fig.1).

Reducing Power Assay

The values obtained for the partially purified extract in comparison with the standard ascorbic acid are presented in Fig. 2. As is evident from the graph, reducing power of the extract is significantly low as compared to the standard.

Nitric Oxide Scavenging Activity

About 37.90% of nitric oxide scavenging was obtained with the partially purified earth worm extract as depicted in Fig. 3.

Anti-inflammatory Activity

The different concentrations of earthworm extract were analyzed for, along with Diclofenac Sodium as standard, % of the stabilization of RBC membrane after pre-treatment with hyposaline buffers.The crude protein of 30mg/ml stock concentration was employed and significantly high stabilization of RBC membranes was noticed. Pretreated RBC in hyposaline buffer were incubated with concentration of 50µg/ml -2000µg/ml of Diclofenac Sodium and earthworm extract and % of hemolysis was noticed in the range of 81.44- 69.90 and 76.60-41.40 for the drug and the extract respectively. Thus results from in-vitro analysis confirmed strong anti-inflammatory activity of earthworm protease.

DISCUSSION

Phenolic compounds are known as powerful antioxidants because of their chain breaking ability attributed to their hydroxyl groups. Polyphenolic compounds which are important plant constituents are known to have inhibitory effects on mutagenesis and carcinogenesis in humans when ingested up to 1 g daily from a diet rich in fruits and vegetables. Ethanolic extract of *Adiantum lunulatum* is reported to show strong antioxidant activity by inhibiting DPPH, Hydroxyl, H_2O_2 and nitric oxide radicals, and reducing power activities when compared with standard ascorbic acid. The plant extract is also reported to contain considerable quantities of total phenols [11]**.** The results of our study indicate a total phenolic content of 8.80 µg GAE/mg reflecting the presence of amino acids tyrosine and phenylalanine in the crude protein isolate of the extract.

Protein hydrolysate from plants as well as animals was found to show antioxidant property and was reported to depend on the enzymes employed during oxidation [26]**.** Different methods used for measuring antioxidant activity based on different mechanisms may lead to different observations [27]. To understand the antioxidant activity better, total phenolic content was estimated along with reducing power assay and Nitric oxide scavenging activity. Study on anti-oxidant activity of silk worm larvae protein isolate (SLPI) and gastrointestinal hydrolysate [12] indicated that the SLPI has a relatively higher DPPH radical scavenging activity, reducing power and ferrous ions chelating capacity. Hence it is suggested that it could be used

as a promising protein resource for preparation of the biopeptides with ACE-inhibitory and anti-oxidant activities.

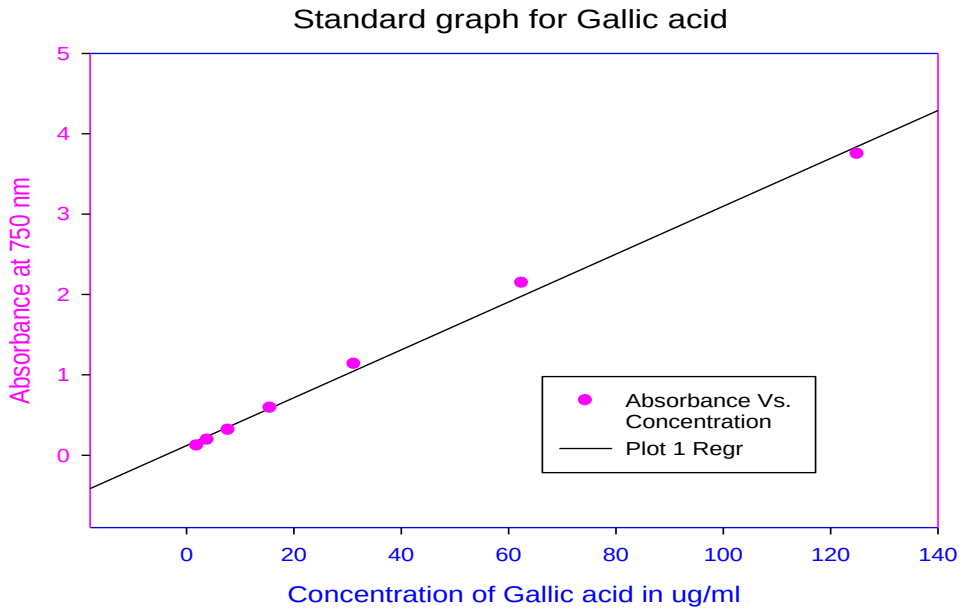
A study on the effect of administration of the earthworm extract in rats demonstrated a significant increase in the activities of antioxidant enzymes SOD and CAT which diminished by Paracetamol [19] and CCl_4 [20] treatment. In these studies, GSH content was found to be significantly enhanced after treatment with earthworm extract, confirming its capacity of scavenging free radicals. It was suggested that the cellular mechanisms to prevent the oxidative stress damage by Earthworm Extract is by the modulation of the genes of antioxidant enzymes such as SOD and CAT [6, 7]. Our results indicate that the autolysed extract of the earth worm *Pheretima posthuma* has considerable nitric oxide scavenging (37.9%) and reducing power ability, although less in comparison with the standard, Ascorbic acid. The protocols employed in the study have certain chemical reagents like Sodium azide which are strong protein inhibitors. Hence, it is opined that the actual anti-oxidant property of the extract would be more than what the present results suggest.

Serine protease has been reported for excellent Anti-inflammatory activity isolated from different sources and HRBC is the best method for in-vitro determination of % of RBC of hemolysis and % RBC membrane stabilization [28]. The partially purified protein extracts of *Pheretima posthuma* showed very significant membrane stabilization activity (58.6%) as compared to the drug Diclofenac sodium (30.1%) suggesting that this earthworm protein should be studied further to explore precisely the protein fraction responsible for this activity. Our experimental results are in corroboration with the findings of Omar et al., 2012 [20] from their experiments on rats with the extracts of two earthworms viz., *Pheretima hawayana* Rosa and *Allolobophora caliginosa* Savigny.

The earthworm protease, a group of 6-8 isoforms, exhibits diverse therapeutic properties along with anti-inflammatory activity. The exact molecular mechanism of all the isoforms with distinct therapeutic property is not explored yet completely but many speculations have been made based on preliminary investigations [20]. The most accepted hypothesis for anti-inflammatory activity of earthworm protease is that it is cyto-protective and neutralizes inflammatory mediators at an early stage and further is facilitated by strong antioxidant activity [10]. Further, it is reported that serine proteases regulate various physiological process like inflammation, blood coagulation, Mast cell physiology and allergic response by stimulating or inhibiting protease-activated receptors (PARs) [29]. There are numerous studies carried out with crude extracts of the whole body or coelomic fluid for finding anti-inflammatory potential of different species of earthworms. The major challenge is finding an exact molecular mechanism of action of earthworm protease as it exists in isoforms of 6- 8 proteins with different molecular weights. Therefore, It is essential to go for ultra-purification in order to reveal the complete molecular etiology of earthworm proteases.

ISSN: 0975-8585

Figure 1: Standard graph for Gallic acid (Absorbance (750 nm) Vs Concentration in µg/ml)



Total Phenolic content is estimated to be 8.80 µg GAE/mg

Reducing Power of the Sample (EE) in comparison with the Standard (Ascorbic acid)

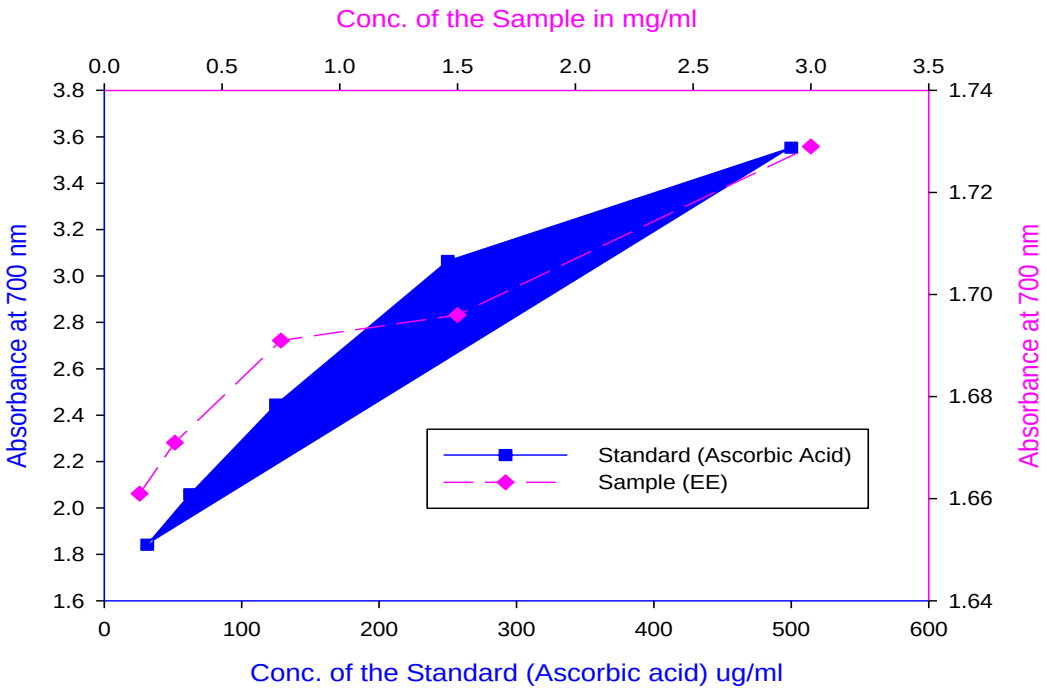


Figure 2

ISSN: 0975-8585

Figure 3

Nitric Oxide Scavenging Activity of the Sample in comparison to the Standard, Ascorbic acid

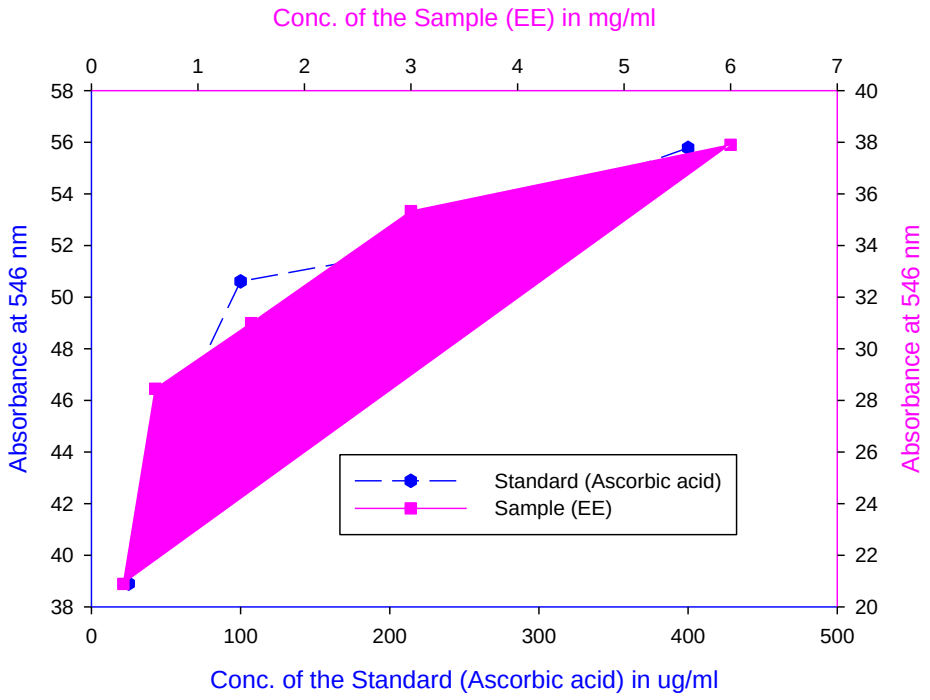
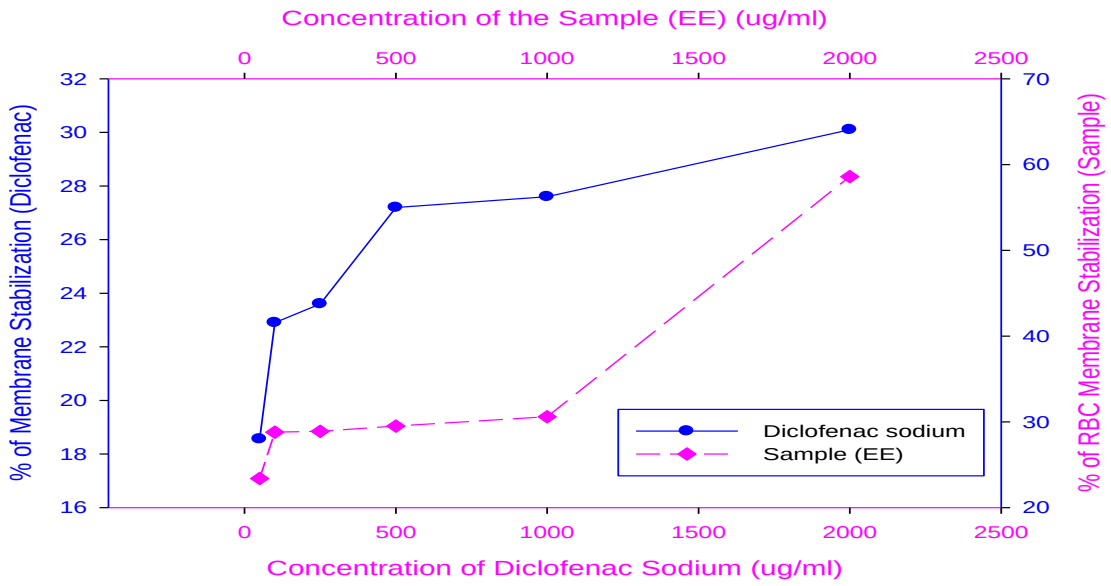


Figure 4

% of RBC Membrane Stabilization - a comparison between the drug and the sample



ISSN: 0975-8585

ACKNOWLEDGEMENTS

We express our deep sense of gratitude to the Management and the Principal, R.V.R. & J.C. College of Engineering, Guntur, Andhra Pradesh, India for providing facilities to carry out the present work. This work has been supported by the Research Grant for Faculty, sanctioned to the corresponding author with the Grant No. R&J/Budget/2011-12/251.

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