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Investigation of *In Vitro* Anti-Ulcer, Anti-Diabetic, and *Ex Vivo* Anti-Inflammatory Activities of Natural Compound Esculin

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Abstract: Natural components are gaining popularity nowadays due to their ease of availability and effectiveness in treatment of various ailments. Esculin is one of such natural components, which exhibits numerous pharmacological properties including anti-cancer, antibiotic, anti-viral, neuroprotection, anti-thrombosis, anti-ulcer, anti-diabetic, anti-oxidative stress, anti-inflammatory and treating eye diseases. The present work aimed to evaluate the anti-ulcer, anti-diabetic, and anti-inflammatory effects of Esculin using *in vitro* and *ex vivo* techniques which have not been explored yet. *In vitro* anti-ulcer activity and *in vitro* anti-diabetic activity were assessed by using artificial gastric acid method and glucose uptake inhibition methods, respectively. Protein denaturation by egg albumin and heat induced hemolysis methods were performed *ex vivo* to assess the anti-inflammatory activity of esculin. Esculin showed a significant acid neutralising capacity in dose dependant manner (100, 150, 200 and 250 mg) and inhibition of glucose uptake at various doses (25 mg/ml and 50 mg/ml) was also observed in comparison to standard drugs. Esculin showed reduction in protein denaturation and thermal degradation at various doses (100, 200, 300, 400 and 500 µg/ml) which revealed better anti-inflammatory activity. This study demonstrated the possible pharmacological activities of esculin as a lead compound.

Keywords: Esculin, Anti-ulcer, Anti-diabetic, Anti-inflammatory, Glucose uptake

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Introduction

Plants possess an abundance of diverse compounds, among which numerous are secondary metabolites. These compounds encompass some extensive substances which are

aromatic in nature, predominantly carbolic acid and their derivatives like tannins. It's noteworthy that a significant portion of these compounds exhibit remarkable antioxidant properties (Galer

and Benzie, 2011). As mentioned in the World Health Organization (WHO), a medicinal plant is defined as a plant that possesses constituents in any one of its parts that can be utilized for medicinal purposes or serve as messenger for chemical or pharmacological semi-synthesis (Ghosh *et al.*, 2023). Novel biologically active natural products will persist their crucial role as lead compounds for drug development and also act as biochemical probes for the exploration of pharmacological and biochemical processes. In drug development process time, money and toxicity are the major obstacles. They can be overcome by using conventional methods and experiential database to collect novel functional leads.

Esculin is a hydroxycoumarin which is the 6-O- β -D-glucoside of esculetin. It acts as an antioxidant agent and also as a metabolite agent. It is a β -D-glucoside and a hydroxycoumarin. It is functionally related to an esculetin. Esculin is found in barley. Esculin is a glucoside agent which is present in natural plant sources like horse chestnut (*Aesculus hippocastanum*), California buck eye (*Aesculus californica*) and in daphne (*Daphne mezereum*) (Li *et al.*, 2022). Several studies reported that esculin is favorable to be used to treat a number of diseases related to inflammation and oxidative damage. Esculin's major actions are improving capillary permeability and perivascular connective tissues integrity by suppressing catabolic enzymes like hyaluronidase and collagenase. Esculin also exhibited strong antioxidant capabilities, shielding triglycerides from auto-oxidation at high temperatures. The anti-inflammatory activity is due to its antioxidant property (Arulselvan *et al.*, 2016).

Ulcers are open wounds or lesions that develop on the outer surface of skin or mucous membranes. Gastric and duodenal ulcers result from erosion of the thick inner lining of the stomach and duodenum, often due to *Helicobacter pylori* infection or chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs). If untreated, ulcers

can lead to complications such as infection, tissue necrosis, cellulitis, sepsis, and, in chronic cases, even malignancy (Prabhu and Shivani, 2014). Diabetes is a chronic metabolic disorder which is either due to pancreas not producing enough insulin or the cells of the body not responding to the insulin produced (Kharroubi and Darwish, 2015). Inflammation is a defense mechanism of immune system in response to injurious stimuli, such as microbes, tissue damage, and irritants. It is an essential process aimed at protecting and repairing the damaged tissues. Chronic inflammation can lead to wound formation, impaired organ function, and an imbalance in the body's immune system (Kumar *et al.*, 2011).

The prime objective of the study was to assess the anti-ulcer, anti-inflammatory and anti-diabetic effects of Esculin using *in vitro* and *ex vivo* techniques. Initially, the study aims to assess the *in-vitro* anti-ulcer activity of esculin by testing its effectiveness against artificial gastric acid. Furthermore, the study seeks to examine the *in vitro* anti-diabetic activity of esculin through the glucose uptake inhibition by yeast cells technique. Then the anti-inflammatory activity of esculin was evaluated by *ex vivo* protein denaturation and heat induced haemolysis techniques. By utilizing *in vitro* and *ex vivo* techniques, this research aims to provide valuable insights into the potential therapeutic benefits of Esculin for managing ulcers, treating diabetes and addressing inflammation related conditions.

Materials and Methods

Materials:

Glucose, Sodium bicarbonate, Yeast and Dimethyl sulphoxide were procured from the Fischer Scientific Pvt. Ltd., Mumbai, India. Aurobindo Pharma Ltd., Hyderabad, India provided the gift samples of Glimepiride and Ibuprofen. Aluminium hydroxide, Magnesium hydroxide (Gelusil) and Esculin were purchased from Pfizer Limited., Mumbai, India.

In vitro Anti-ulcer activity:

Neutralizing effect of artificial gastric acid:

The acid neutralizing capacity value for Esculin at various doses i.e., 100, 150, 200 and 250 mg was correlated with the standard antacid aluminium trihydroxide and magnesium dihydroxide mixture (500 mg). Water was added to 5 ml quantity of this mixture and then make up the volume to 70 ml then mixed for 1 min. Subsequently, standard and test preparations were mixed for 15 min with 30 ml of 1N hydrochloric acid (HCl). After mixing, a few drops of phenolphthalein solution were added. As soon as possible, the surplus HCl was titrated drop by drop with 0.5N sodium hydroxide solution until a pink hue was achieved (Umre *et al.*, 2018). The results are demonstrated in Table 1. The moles of acid neutralized and acid neutralizing capacity (ANC) were estimated using the following formulae:

$$\begin{aligned} \text{Moles of acid neutralized} &= (\text{Volume of HCl} \times N \text{ of HCl}) - (\text{Volume of NaOH} \times N \text{ of NaOH}) \\ \text{Acid neutralizing capacity} &= \frac{\text{Moles of HCl neutralized}}{\text{Grams of antacid (Extract)}} \end{aligned}$$

In Vitro Anti-Diabetic Activity:

Glucose uptake Inhibition in Yeast cells:

Saccharomyces cerevisiae, also known as baker's yeast at a quantity of 1g was mixed using distilled water and centrifuged at a speed of 3000 rpm for 5 min, until a clear supernatant was observed. From this, by adding distilled water a 10% v/v suspension was prepared. Esculin, at a pair of concentrations (25 and 50 mg/ml) were dissolved in Di Methyl Sulphoxide (DMSO) and mixed to 1 ml of glucose solution prepared at varying concentrations of 5, 10 and 20 mM and incubated for 10 min at 37°C. 100 µl of yeast suspension was added to start the reaction after 10 min, and it was then vortexed and incubated for an additional 60 min at 37°C. The tubes were centrifuged at 2500 rpm for 5 min after 60 min, and the amount of glucose in the supernatant was calculated (Rehman *et al.*, 2018). The results are demonstrated in Figures 1 and 2. Glimepiride was referred as standard drug. By using the given formula, the percentage increase in glucose uptake by yeast cells was calculated:

$$\text{Increase in glucose uptake (\%)} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100$$

Where, Abs_{sample} is test sample absorbance; Abs_{control} is control absorbance.

Ex vivo Anti-Inflammatory Activity:

The *ex vivo* anti-inflammatory effect was estimated through two different methods:

Egg Albumin Method to achieve Protein Denaturation: The reaction mixture (5 ml) was put into test tubes. It contained 0.2 ml of egg albumin, 2.8 ml of phosphate-buffered saline (PBS, pH 6.4), and 2 ml of Esculin at different concentrations (100, 200, 300, 400, and 500 µg/ml). The control was an equivalent volume of double-distilled water. The mixtures were heated to 70°C for 5 min after being incubated for 15 min at 37 ± 2°C in a BOD (Biological Oxygen Demand) incubator. After cooling, the samples' absorbance was measured at 660 nm by using the vehicle as a blank. To determine absorbance, ibuprofen at concentrations of 100, 200, 300, 400, and 500 µg/ml was used as a standard and handled in the same way (Elias and Rao, 1988). The results are demonstrated in Figure 3. By the given formula, the percentage inhibition of protein denaturation was measured:

$$\% \text{ Inhibition} = 100 \times \left[1 - \frac{A_2}{A_1}\right]$$

Where, A₂= absorbance of test sample, A₁= absorbance of control.

Heat Induced Hemolysis Method: The technique of stabilising RBC membranes was used to measure the anti-inflammatory activity. Rats were given fresh blood, which was combined with an equivalent amount of Alsever solution and centrifuged for 10 min at 3,000 rpm. After using iso saline to wash the packed cells, a 10% solution was created.

Using distilled water, several concentrations of esculin (10, 20, 30, 40, and 50 µg/ml) were made. 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 ml of RBC suspension were added to each concentration. They underwent a 30 min

incubation period at 37°C and a 20 min centrifugation at 3,000 rpm. A spectrophotometer set to 560 nm was used to measure the amount of haemoglobin in the supernatant solution. The standard for comparison was ibuprofen (100 mg/ml), and a control was made by leaving out the extracts. Three duplicates of the experiments were run, and the mean values were taken into consideration (Shinde *et al.*, 1999). The results are demonstrated in Figure 4. By using the following formula, the percentage (%) of Human red blood cell membrane stabilization (HRBC) or protection was calculated:

$$\%Protection = 100 - \left(\frac{OD \text{ of drug treated sample}}{OD \text{ of control}} \right) \times 100$$

Where, OD indicates optical density.

Statistical Analysis:

All the statistics obtained were expressed as mean $\pm$ standard error of mean (SEM). The data was statistically analyzed by utilizing Graph pad prism software (version 5.0).

Results and Discussion

In the present study, Esculin a natural compound which is popular in traditional medicine was screened for several pharmacological actions through *in vitro* and *ex vivo* techniques. The commercially procured Esculin was subjected for *in vitro* anti-ulcer, anti-diabetic and *ex vivo* anti-inflammatory activities. After evaluation of various pharmacological actions, the obtained results were correlated with that of the reference drug to know the efficacy of Esculin.

Anti-ulcer activity:

Acidity is a characteristic gastrointestinal problem among people of all ages. Antacids acts by neutralizing the gastric acid, which increases the pH of gastric acid. The amount of acid that can be neutralized by an antacid is the acid neutralizing capacity, which can be measured using back titration method. Medicinal herbs offer a rich source of numerous chemical compounds that exhibit potent antiulcer activity. Those phytochemicals can be developed into herbal

drugs by using modern technology with less side effects (Koka *et al.*, 2022). Esculin at highest concentration of 250 mg showed a significant reduction in acid neutralising capacity of 35.87 ± 0.68 . Similarly, the volume of sodium hydroxide consumed and mEq of acid consumed were also less for esculin at a dose of 250 mg. The standard $Al(OH)_3 + Mg(OH)_2$ at a dose of 500 mg showed ANC of 32.52 ± 0.51 . This shows the antacid and anti-ulcer property of the esculin. The results are given in Table 1.

Anti-diabetic activity:

When blood insulin secretion is elevated, leptocytes and monocytes regulate the molecules that transport glucose, producing a hypoglycemic effect (Tan *et al.*, 2019). In general, most of the studies focused on the impact of medications on the decrease of postprandial hyperglycemia, which is one of the key elements in the treatment of diabetic mellitus. Moreover, yeast cells may not absorb glucose in the same way as other eukaryotic or human body cells (Tijjani and Imam, 2021). Glucose transport across the membrane of yeast cells could be due to mediation of phosphotransferase enzyme rather the process of facilitated diffusion or may be due to any other unknown mechanism. Glucose Uptake by yeast cells might be affected by many components like amount of glucose inside the cells or by glucose metabolism (Cirillo, 1962).

Esculin effectively decreased the percentage inhibition of glucose uptake at two doses of 25 and 50 mg/ml which is almost nearer to the standard drug Glimepiride. This might be due to the effective binding of Esculin to glucose and transporting it across the cell membrane to facilitate metabolism. This proves the anti-diabetic effect of esculin. Examining Esculin's anti-diabetic action *in vivo* is bound to be captivating, as it may contribute to improved glucose absorption by muscle and fat tissues. The results are demonstrated in Figures 1 and 2.

Anti-inflammatory activity:

Fresh egg albumin was used to treat esculin to

Table 1: Neutralizing Effect of Esculin on Artificial Gastric Acid

S. No.	Dose (mg)	Volume of NaOH consumed (ml)	mEq of acid consumed	ANC per gram of antacid
1	50	45.84±1.08	9.94±0.45	182.05±2.58
2	100	44.15±1.11	9.66±0.66	99.51±1.61
3	150	42.36±1.01	9.35±0.59	62.33±0.72
4	200	37.44±0.95	8.31±0.55	41.53±0.54
5	250	35.19±1.25	8.05±0.48	35.87±0.68
6	AlOH & MgOH (500)	27.53±1.05	6.25±0.63	32.52±0.51

Mean ± Standard Error of Mean (SEM) N = Three samples

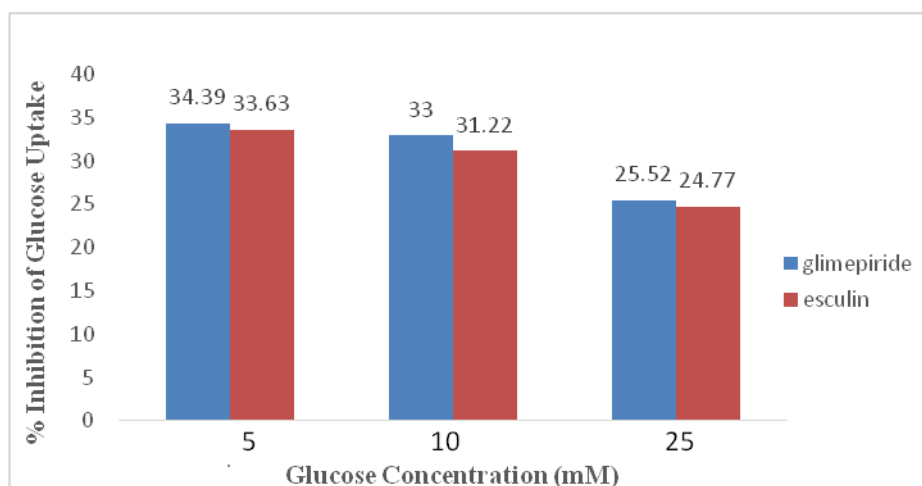


Fig. 1: Effect of Glimepiride and Esculin on %Inhibition of Glucose Uptake (25 mg/ml).

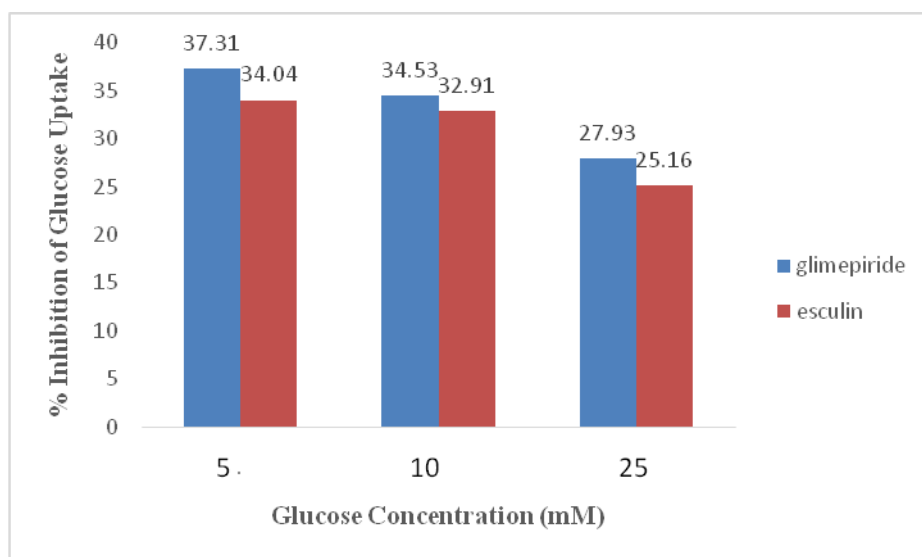


Fig. 2: Effect of Glimepiride and Esculin on % Inhibition of Glucose Uptake (50 mg/ml).

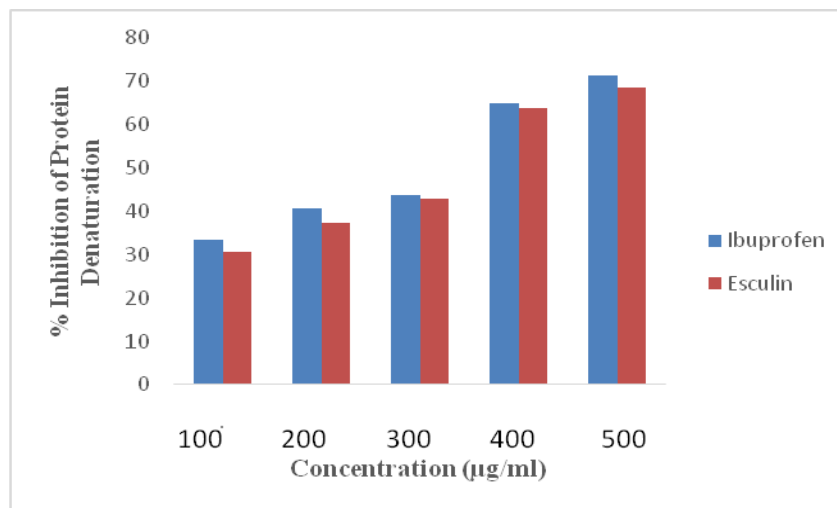


Fig. 3: Effect of Esculin on Protein Denaturation.

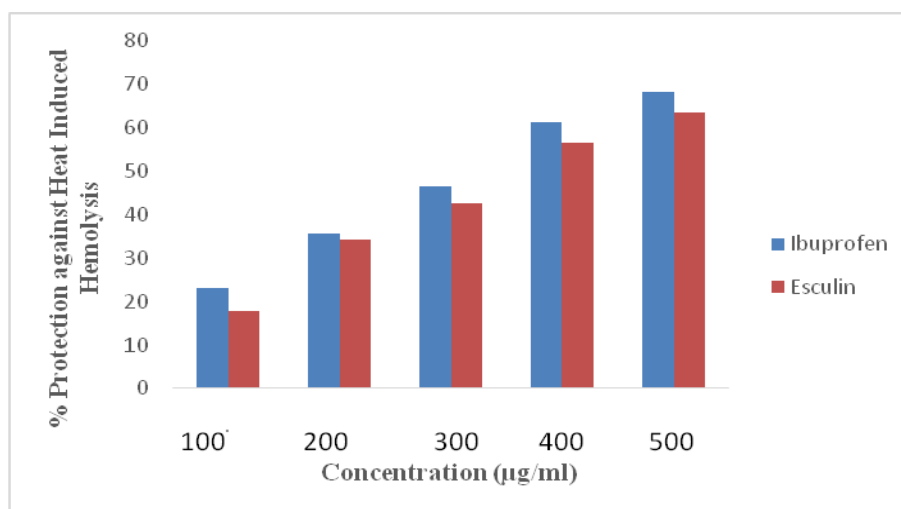


Fig. 4: Effect of Esculin on Erythrocyte Membrane Stability.

protein denaturation at different concentrations. At a maximum dose of 500 µg/ml, esculin demonstrated 68.47% suppression of protein denaturation. When compared to the standard, it is just as effective, and the percentage of protein denaturation inhibition rises with increasing dose. Many anti-inflammatory agents exhibited dose dependent activity to reduce heat induced denaturation of protein (Lakheda *et al.*, 2011). Esculin's capacity to reduce heat-induced protein denaturation may be a contributing element in its anti-inflammatory properties. The results are shown in Figure 3.

Using fresh rat blood, esculin was tested for

heat-induced hemolysis at different doses. At a maximum dose of 500 µg/ml, esculin demonstrated 63.56% protection against heat-induced hemolysis of erythrocyte membrane. When compared to the standard, it is nearly as effective, and the percentage of red blood cells protected rises with increasing dosage (Doppalapudi *et al.*, 2012). The integrity of their membrane determines the vitality of cells. When red blood cells are exposed to harmful materials like hypotonic solution, it leads to membrane lysis along with haemolysis and haemoglobin oxidation (Siddik *et al.*, 2021). This could be the possible mechanism behind the protective activity of Esculin. The results are demonstrated in Figure 4.

Conclusion

The current study's findings led to the conclusion that Esculin at concentration 250 mg showed significant anti-ulcer activity, reduction of glucose uptake at 25 and 50 mg/ml which showed anti-diabetic activity and at concentration of 500 µg/ml showed anti-inflammatory activity in *in vitro* and *ex vivo* modes. To evaluate its clear mechanism of action and *in-vivo* efficacy, further studies are needed.

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