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EVALUATION OF *IN-VITRO* ANTI-INFLAMMATORY AND ANTICOAGULANT ACTIVITIES ON SYNTHETIC COMPOUNDS COUMARIN AND CURCUMIN

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ABSTRACT

The present study was aimed to evaluate the *in-vitro* anti-inflammatory and anticoagulant activities on synthetic compounds Coumarin and Curcumin. The *in-vitro* anti-inflammatory activity was carried out by using protein denaturation and HRBC membrane stabilization methods. In this evaluation found that synthetic product coumarin and curcumin derivative **(10, 25, 50 µg/ml)** had shown good anti-inflammatory activity when compared to that of standard Diclofenac sodium. This activity is due to the presence of chromene nucleus and the curcumin nucleus present in these compounds.

The anticoagulant activity was performed by estimating the prothrombin time for fresh human blood. In this evaluation found that Test sample **(10, 25, 50 µg/ml)** on increasing concentration the time of coagulation also increased, also found that test sample high anticoagulant activity than the 0.9%Nacl.From the study it was concluded that coumarin and curcumin derivative has good *in-vitro* anti-inflammatory and anticoagulant activities by performing the protein denaturation and HRBC membrane stabilization for anti-inflammatory and prothrombin time for anticoagulant activity methods.

INTRODUCTION

Inflammation is closely linked with other physiological systems including the coagulation-fibronolytic system and oxidantantioxidant pathways [1]. Physiologically, the activation of inflammation and the coagulation system in acute trauma is recognized as a physiologic reaction to initiate healing and to act as a barrier to injury propagation and infection [2]. Systemic inflammation will invariably lead to activation of the coagulation system, but vice versa, components of the coagulation system can markedly modulate the inflammatory response [3]. Pathophysiologically, inflammation and the coagulation system play crucial roles in the pathogenesis of multiple chronic inflammatory disorders [4].

A blood clot (thrombus) developed in the circulatory system due to failure of hemostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases such as myocardial or cerebral infarction, at times leading to death.[5] Thrombolytic agents that include tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) etc. are used all over the world for the treatment of these diseases. In India, though SK and UK are widely used due to lower cost,[6,7] as compared to other thrombolytic drugs, their use is associated with hyper risk of hemorrhage[8] severe anaphylactic reaction and lacks specificity. Moreover, as a result of immunogenicity multiple treatments with SK in a given patient are restricted[9]. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs [10 – 11].

MATERIALS AND METHODS:

Chemicals and reagents:

All reagents were purchased from Merck and Avra synthesis Pvt.Ltd and used without further purification. 4-hydroxy coumarin, phosphoryltrichloride, curcumin, ethanol, triethylamine, Tri sodium citrate, calcium chloride, sodium chloride, Diclofenac sodium, citric acid, Dextrose, Bovine serum albumin, phosphate buffer, 1N hydrochloric acid.

Requirements:

The instruments used were double beam UV-visible spectrophotometer (ELICO-SL210), centrifuge machine, Shimadzu electronic balance, vacuum filter, kshitij melting point apparatus. Test tubes, centrifuge machine, 5ml syringes, incubator, volumetric flasks, test tubes, measuring cylinder.

General procedure for synthesis:

- The chloraldehyde which is made reacted with curcumin in 0.01 molar ratio.
- Chloraldehyde of Mol.wt 208.6 is prepared from formylation of coumarin nucleus.

- A solution of 4-chloro-3-formyl coumarin (1mmol), curcumin (1mmol) and triethylamine (1 equivalent) in 5ml ethanol were stirred at room temperature for 15 min.
- After completion of the reaction the precipitate obtained was filtered and washed thoroughly with absolute ethanol and re-crystallized from ethanol to afford pure product.

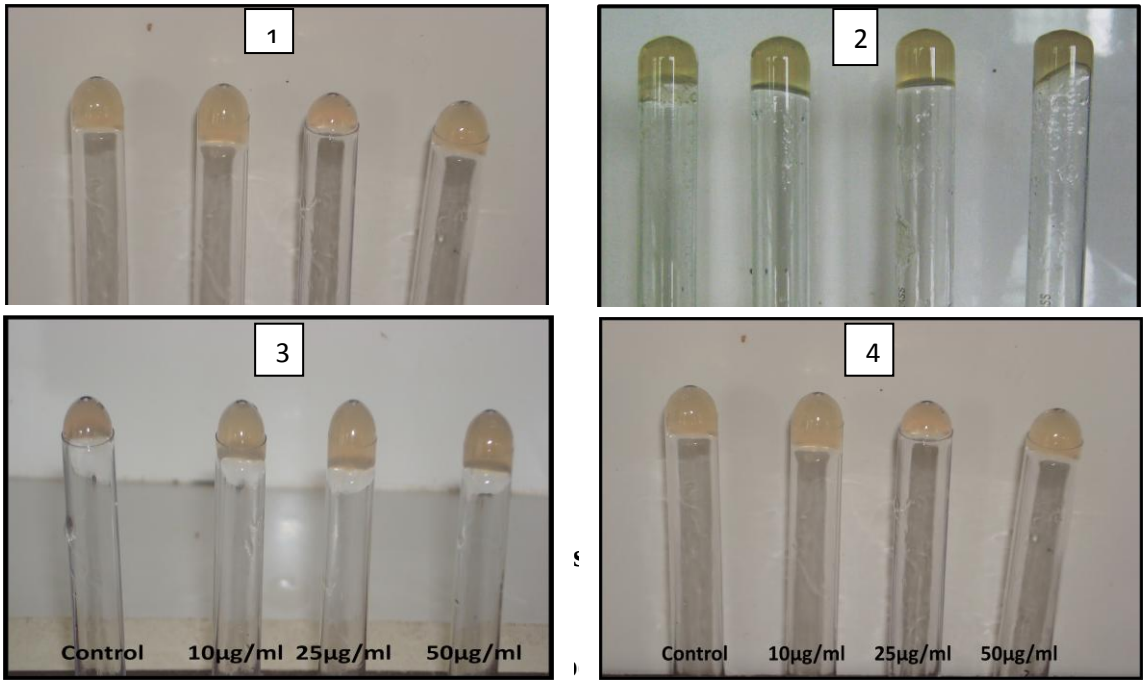
ANTICOAGULANT ACTIVITY:

- **Study Population:**

Blood Samples were collected from the healthy volunteer's age between 20-25 years to assess the anticoagulant effect of the synthesized compounds. The blood samples were withdrawn using sterile syringes from vein of right arm of each individual and placed separately in containers containing tri-sodium citrate to prevent the clotting process. Centrifugation (15 minutes at rate 3000 rpm) was carried out to separate the blood cells from plasma in order to obtain pure platelet plasma (ppp) for prothrombin time test. The obtained plasma sample of each individual was used immediately [12].

Plasma recalcification:

0.2ml plasma, 0.1 ml of different concentrations of the test sample (10µg/ml, 25µg/ml, 50µg/ml) and 0.3ml of $Cacl_2$ (25mM) were added together in a clean test tube and incubated at 37°C. For control experiment test sample was replaced by same volume of 0.9% saline water. The clotting time was recorded with a stopwatch by tilting the test tube for every 5 seconds. This time is called the prothrombin time [13].



The anti-inflammatory activity of Enicostemma axillare was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al [14] followed with minor modifications.

Test solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of test samples of different concentrations (10µg/ml, 25µg/ml, 50µg/ml, and 100µg/ml).Test control solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control solution (0.5 ml) consists of 0.45ml of distilled water and 0.05ml of test samples of different concentrations (10µg/ml, 25µg/ml, 50µg/ml, and 100µg/ml). Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of different concentrations (100µg/ml, 200µg/ml) of Diclofenac sodium.

All the above solutions were adjusted to P^H 6.3 using 1N hydrochloric acid. The samples were incubated at 37°C for 20 min and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, 2.5ml of phosphate buffer was added to the above solutions .The absorbance was measured using UV Visible Spectrophotometer at 416nm. The percentage inhibition of protein denaturation was calculated as,

$$\textbf{\% Inhibition of protein denaturation} = 100 - \left[\frac{(\text{O.D of test solution} - \text{O.D product control})}{(\text{O.D of test control})}\right] \times 100$$

The control represents 100% protein denaturation. The results were compared with Diclofenac sodium.

2. HRBC Membrane Stabilization Method:

The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. Therefore as the membrane stabilizes, it interferes with the release and/or action of mediators like histamine, serotonin, prostaglandins, and leukotrienes which are responsible for inflammation. The prevention of hypotonicity induced HRBC membrane lysis is taken as a measure of Anti inflammatory activity [15].

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.

Procedure (hypotonic solution-induced haemolysis)*:*

The reaction mixture (4.5 ml) consists of 2ml of hyposaline (0.25% w/v NaCl), 1 ml of 0.15 M phosphate buffer (pH 7.4) and 1 ml of test solution (10 µg/ml, 25 µg/ml,50µg/ml,100 µg/ml) in isosaline, 0.5 ml of 10% HRBC in isosaline was added . For test control, 1 ml of distilled water used instead of hyposaline (to produce 100% hemolysis), while product control lacked red blood cells. The mixtures were incubated at 37^0C for 30 min and centrifuged at 3,000 rpm 20 min. Diclofenac sodium was used as the reference drug. The haemoglobin content in the suspension was estimated using a spectrophotometer at 560 nm[16]. Percentage membrane stabilizing activity was calculated as follows,

% Membrane stabilization = 100 – [(OD of Test sample / OD of Control) X 100]

RESULTS:

Anticoagulant activity:

Table no.1: Anticoagulant activity of coumarin and curcumin derivative

Sample	**Concentration (µg/ml)**	**Time of Coagulation(min) for 4 different Individuals**			
		1	**2**	**3**	**4**
Control	**0.9%Nacl**	8:00	10:00	8:04	09:00
Test Sample	**10**	10:00	11:00	10:05	8:30
	25	19:00	24:00	23:00	15:10
	50	39:00	56:00	50:00	32:00

The table no.1 contains time of coagulation of blood for four different individuals. the anticoagulant activity was evaluated by prothrobin time for fresh human blood. In this evaluation found that Test sample **(10, 25, 50 µg/ml)** on increasing concentration the time of coagulation also increased, also found that test sample high anticoagulant activity than the 0.9%Nacl.

ANTI-INFLAMMATORY ACTIVITY

Table no. 2: Inhibition of Protein denaturation method of test sample and Diclofenac Sodium

Concentration (µg/ml)	**Percentage Inhibition of Protein denaturation±SEM**	
	Test Sample	**Diclofenac sodium**
10	39.1±0.41	-
25	52.9±0.24	-
50	70.0±0.129	-
100	75.9±0.082	88.89±0.036
200	-	93.34±0.092

Values are expressed as mean±SEM, n=3

Table no.3: HRBC Membrane Stabilization Method of test sample and Diclofenac Sodium

Concentration (µg/ml)	Percentage Membrane stabilization±SEM	
	Test Sample	Diclofenac sodium
10	59.26±0.143	-
25	65.78±0.086	-
50	75.88±0.135	-
100	81.12±0.167	86.79±0.043
200	-	89.78±0.087

Values are expressed as mean±SEM, n=3

The table no.2 and 3 contains the percentage of anti-inflammatory activity by **HRBC**membrane stabilization method and protein denatuaration methods .The valuaes are obtained by taking the test samples (10,25,50,100,200 **µg/ml)and then see the obsorbence in U.V spectroscopy at 560nm with diclofenac used as standard drug .**then the percentage of inhibition of inflammation was calculated by using the formula

%Membrane stabilization =100-[(OD of test sample / OD of Control) ×100]

DISCUSSION

The data presented in this study demonstrate that synthetic product of coumarin and curcumin posses anticoagulant, anti–inflammatory activities. Curcumin have the anti-inflammatory and anti coagulant activity and when this coumpound incorporated in coumarin nucleus which does not disturb the coumarin activity more over it will increase the coumarin anti inflammatory and anti coagulant activities. The anticoagulant activity was performed by estimating the prothrombin time for fresh human blood [13]. From the present study, it was proved that the synthetic product coumarin and curcumin had shown good anti-coagulant activity than the control solution. The results of Anticoagulant activity was shown in **table no.1,** this activity is due to the curcumin nucleus. Curcumin incorporates several functional groups. The aromatic ring systems, which are phenols, are connected by two α,β-unsaturated carbonyl groups. The diketones form stable enols and are readily deprotonated to form enolates thus curcumin can incorporate coumarin nucleus and both will give increased anti- coagulant activity.

The Anti-inflammatory activity was performed by inhibition of protein denaturation and HRBC (Human Red Blood Cell) Membrane Stabilization methods [14, 15]. From the present study it was proved that synthetic product coumarin and curcumin had shown good anti-inflammatory activity when compared to that of standard Diclofenac sodium. The results was shown in table 2 by inhibition of protein denaturation method and table no.3 by HRBC membrane stabilization methods [16] .This activity is due to the presence of chromene nucleus and the curcumin nucleus.

CONCLUSION

From the study it was concluded that coumarin and curcumin derivative has good *in-vitro* anti-inflammatory and anticoagulant activities by performing the protein denaturation and HRBC membrane stabilization for anti-inflammatory and prothrombin time for anticoagulant activity methods.

The *in-vitro* activities obtained demonstrated valuable indications on a result, further *in-vivo* studies can be taken up to evaluate the different biological activities.

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