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The RBC membrane stablisation in an in vitro method by the drug isolated from *Leucas aspera*

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Abstract: Among the Studies of naturally occurring compounds of all kinds, flavonoids chemistry has emerged from the undirected search for new compounds and the establishment of their structures by conventional means and has been increasingly directed into areas of enquiry which biological consideration comes to play an increasingly important role. Medicinal plants constitute an effective source of traditional and modern medicines. The present research work deals with phytochemical and pharmacological studies of *Leucas aspera*. The fresh flowers of *Leucas aspera* have been found to contain Baicalein and Baicalin. The isolated compounds have been duly characterized by chromatographic and hydrolytic study as well as by UV spectral means. The membrane interaction of albino rat RBC with isolated *Leucas aspera* flavonoids by an in vitro method has been studied.

Keywords: *Leucas aspera*, Baicalein, Baicalin, Albino rat, RBC membrane Stabilization.

1. Introduction

One of greatest difficulties confronting the researcher is the paucity of authentic information on the identity, habitat condition of collection and use of medicinal plants. The subject of phytochemistry is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, biosynthesis turnover and metabolism, natural distribution and biological function [1]. Phytochemistry involves the study of flavonoids, alkaloids etc., the research on plants of medicinal importance is growing phenomenally at the international level. Recent estimates suggest that several thousands of plants have been identified with medicinal applications in various cultures [2].

The rich traditional knowledge base countries like India and China in medicinal plants and health care have led to the keen interest by pharmaceutical companies to use this knowledge as a resource for research and development programme in the pursuit of discovering novel drugs. India has a varietals emporium of medicinal plants and it is one of the richest countries in the world in terms of genetic resources of medicinal plants [3]. Flavonoids constitute one of the most characteristic classes of compounds in higher plants. Many flavonoids are easily recognized as flower pigments in most flowering plants. However, their occurrence is not restricted to flowers but includes all parts of the plant. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds mean

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that many animals, including humans, ingest significant amount of flavanoids in their diet. Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity [4]. Hence consumers and food manufactures have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancer and cardiovascular diseases [5]

Leucas aspera (Labiatae) is a variable annual and is distributed more or less throughout India. The leaves are said to be useful in chronic rheumatism. The juice is applied in psoriasis and other chronic skin eruption [6]. The whole plant is used for analgesic–antipyretic, antirheumatic, anti-inflamm atory, and antibacterial treatment, antioxidant activity etc [7-9]. Chemical compounds such as sterols, fatty acids, lactones, long-chain compounds, aliphatic ketol, phenols and alkaloids had been isolated [9-15]. However there have been no reports so far on isolation of flavones from flower of this plant and the study of RBC membrane stabilization using the isolated flavonoid in an in vitro method.

Erythrocytes have been used as a model system by a number of workers for the study of interaction of drugs with membranes [16-18]. Drugs like anesthetics transquilisers and non-steroidal anti-inflamatories stablise erythrocytes against hypotonic haemolysis at low concentration [19]. When the RBC is subjected to hypotonic stress the release of hemoglobin from RBC is prevented by anti-inflammatory agents because of membrane stabilization. So, the stabilization of HRBC membrane by drugs against hypotonicity induced haemolysis serves as a useful in vitro method for assessing the anti-inflammatory activity of various compounds [20]. During this present investigation, the membrane interaction of *Leucas aspera* components has been studied using albino rat blood cells as a model system and the mechanism of hemolytic effect produced by the components has been investigated.

2. Materials and method

Fresh flowers (1 kg) of *Leucas aspera* were collected from Ariyalur district in Tamil Nadu. They were extracted with 90% methanol (4×500ml) under reflex. The alcoholic extract was concentrated *in vacuo* and the aqueous concentrate was successively fractionated with peroxide free ether (3×250ml) and ethyl acetate (4×250ml).

Ether fraction:

The ether fraction was concentrated *in vacuo* and left in the ice chest for about 10 days. It produced colourless crystals (M.P. 265-266°C) were recrystalized from methanol. It was soluble in organic solvent and sparingly in hot water. It resulted a red colour with MgCl, green colour with alcoholic Fe^{3+}, and appeared deep purpled under UV and UV/NH_3. It answered Wilson's Boric acid and Gibbs's test. It did not respond to Horhammer-Hansel and Malisch's tests.

Ethyl Acetate fraction:

The ethyl acetate fraction was concentrated *in vacuo* and left in an ice chest for two days. Yellow solid was separated, filtered and studied. When crystalised from methanol, it came out as pale yellow needles (M.P.335-340°C). It produced green colour with Fe^{3+} and orange red colour precipitated with lead acetate. It dissolved in alkali and NH_3 with a yellow colour that changes into dark brown. It was slightly soluble in hot glacial acetic acid and soluble in ethanol and ethyl acetate, but insoluble in ether and chloroform ($CHCl_3$), which turned deep purple on fuming with NH_3. It answered Wilson's boric acid, Gibb's

and Molisch's tests. But it did not respond to Horhammer-Hansel test.

Membrane stabilisation

Albino rat in RBC was collected in Alsevers's solution (that contains 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride) using sterile 22 gauge hypodermic needle. Blood obtained from albino rat was centrifuged at 3000rpm and the pellet obtained was washed thrice in physiological saline (0.85% pH 7.2) and suspended at 2% (v/v) concentration shortly before use.

The membrane interaction of these isolated drugs (16-18) has been studied using albino rat red blood cells (RBC) as a model system. 0.5 ml of 10% albino rat RBC suspended was added to a solution containing 4ml of 0.25% hyposaline, 1ml of phosphate buffer and 5 μg/ml of the drug. Different doses of the drug had been treated in different tubes. All the tubes were incubated at 37°C for 30 minutes. Then they were centrifuged in a clinical centrifuge and the supernatant hemoglobin content was estimated using klet type calorimeter at 560nm. A control has been done without the drug.

3. Result and Discussion

Hydrolysis of the glycosides:

The glycoside (0.01g) dissolved in hot methanol was hydrolysed with 60% H_2SO_4 at 100°C. For about 3hours the excess alcohol was distilled from the hydrolysate and the resulting aqueous solution was diluted with more water and left under chilled condition for 2hrs.The separated yellow solid was filtered, washed and dried. The aqueous filtrates were extracted with ether. The dry yellow residue on the filter paper was combined with the residue from the dry ether extract and studied for the aglycone. The yellow aglycon on recrystalisation from hot methanol afforded a pale yellow crystalline solid, (M.P. 265-266°C) which was identified as Baicalein by colour reaction, behavior under UV and Rf values (Table 1). Its melting point was analyzed on admixture with an authentic sample of Baicalein from *Oroxylum indicum* [21]. It had the same UV spectral values mentioned under ether fraction. A yellow solid crystalised from methanol came out as pale yellow needles, (M.P. 335-340°C) which was identified as Baicalin analyzed on admixture with an authentic sample obtained from *Oroxylum indicum* [22].

The fresh flowers of *Leucas aspera* have been found to contain Baicalein and its glycoside Baicalin. The UV spectrum of the aglycon shows two major absorption peaks at 323nm (band I) and 274 nm (band II) showing a flavones skeleton. The flavone oxygenated in A-ring, but not in the B-ring, tend to give spectra in methanol with a pronounced band II and a week band I. A bathochromic shift of only 43nm in band I observed in its. NaoMe spectrum indicated the absence of 4'OH group. The $AlCl_3$ / Hcl spectra of the aglycon showed 2 absorption peaks indicating a free 5 OH group. A bathochromic shift of 23 nm is an evidence for the 6-oxygenation pattern along with the free 5OH group. A bathochromic shift of +29 nm on the addition of $AlCl_3$ over and above that of $AlCl_3$/Hcl show on ortho- dihydroxyl grouping in the A-ring. The presence of c-7 OH group was evident from a shift of +10 nm relative to the methanol spectrum. The presence of an ortho- dihydroxyl group in the A-ring could be inferred from the smaller shift of +10 nm on the addition of H_3BO_3.

The UV spectrum of the glycoside showed 2 absorption peaks at 315nm and 278nm. As it was already seen the presence of oxygenation in the A-ring and the absence of the same in the B-ring had been evidenced from the spectra in MeOH with a pronounced band II and weak band I. The absence of a free 4' OH was observed from the absence of any bathochromic shift of band I due to the addition of

NaoMe. As mentioned earlier a smaller bathochromic shift of band I +23 on the addition of $AlCl_3$ showed a free 5 OH with a 6 oxygenation. A bathochromic shift of +33 nm on the addition of NaoAc showed that the 7^{th} position is not at all free. This was evidenced by the NaOAc spectrum of its aglycone a smaller shift of only +3nm on the addition of $NaOAc/H_3BO_3$, showed that A-ring has an ortho-dihydroxyl grouping. Based on the above mentioned physical and chemical evidences the aglycone and glycoside obtained from *Leucas aspera* flowers has been charecterised as the Baicalein and Baicalin respectively.

In general the flavonoids were found to be effective in stabilizing the albino rat RBC membrane against hypotonicity induced hemolysis and hence it would be effective as non steroidal complex anti-inflammatory compounds in the control of inflammation (4, 7-9, 20). Within the experimental range of dosages of (10 to 500μg/ml) the isolated flavones glycoside was observed to be having two maximum values for its activity (Figure 1). At a concentration of 30.5μg/ml the drug is able to stabilize the RBC. But as the concentration is increased the membrane stabilsation once again got dropped to a minimum value at 90 μg/ml. The curve once again moves up to show another maximum value at 210 μg/ml, where the maximum membrane stabilisationis was noticed. Further increase in concentration produced. Only hemolysis and the curve reached equilibrium from 375 μg/ml. The study of albino Rat RBC membrane stabilization using isolated glycoside in an in vitro method, the drug showed a biphasic activity in such a way that at a concentration of 210 μg/ml it observes to be having the maximum membrane stabilization.

Table 1. Rf (x 100) values of the constituents of flowers of *Leucas aspera.* (Whatman No: 1 Ascending, 30 ± 2 c)

COMPOUND	DEVELOPING SOLVENTS							
	a	b	c	d	e	f	g	h
Glucoside from Ethyl Acetate fraction	67	20	34	65	76	51	63	85
Baicalin (authentic)	67	21	34	65	76	50	63	85
Aglycone from the above glycoside	05	07	17	44	69	91	88	76
Baicalein (authentic)	05	07	17	44	69	90	88	76

Solvent key: a- H_2O
b- 5% aq.HOAc
c- 15%aq. HOAc
d- 30% aq. HOAc
e- 60% aq. HOAc
f- n-BuOH: HOAc: H_2O = 4:1:5 (upper phase)
g- Water saturated phenol
h- Forestal

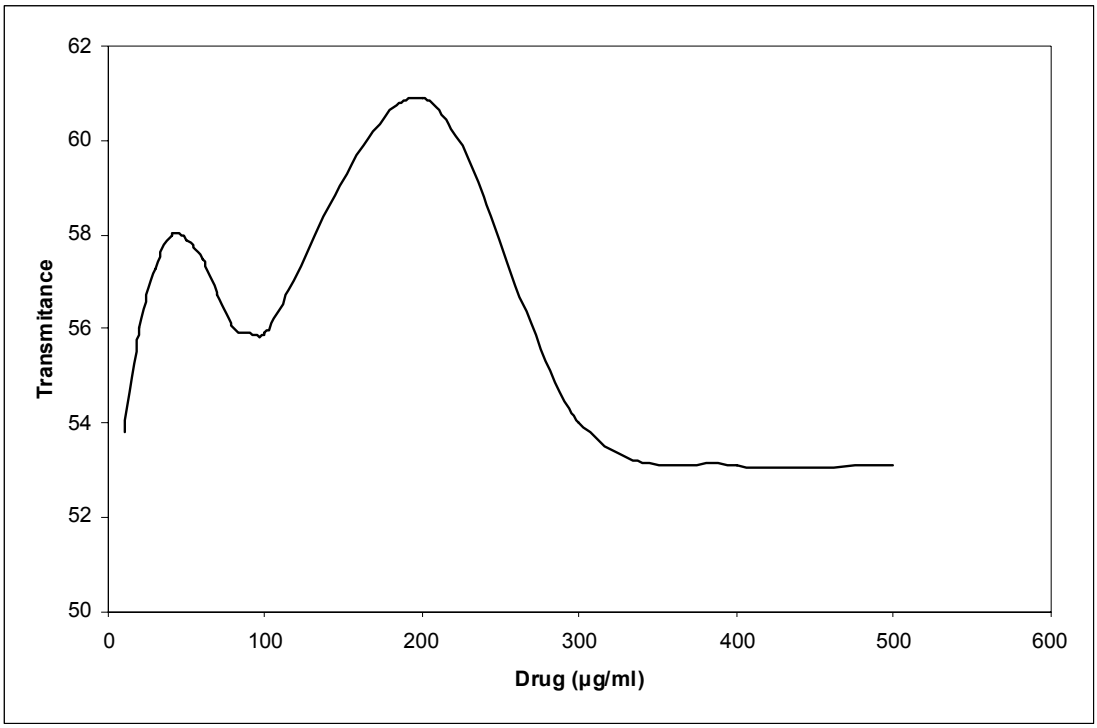

Figure 1. RBC Membrane stabilization by Drug concentration.

4. Conclusion

The membrane interaction of *Leucas aspera* components has been studied using albino rat RBC as a model system and the mechanism of hemolytic effect produced by the components has been investigated. The albino rat RBC membrane against hypotonicity induced hemolysis and hence it would be effective as non- steroidal complex anti-inflammatory compounds in the control of inflammation.

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