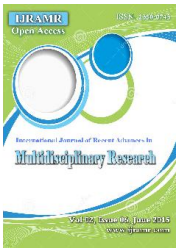

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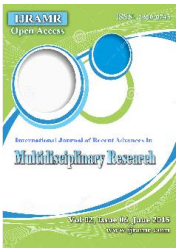

Research Article

IN VITRO EFFECTS OF AQUEOUS EXTRACTS OF FIVE SRI LANKAN MEDICINAL PLANTS ON HUMAN ERYTHROCYTE MEMBRANE STABILISATION ACTIVITY

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ABSTRACT

In vitro assessment of human erythrocyte membrane stabilisation activity of water extracts of *Clitoria ternatea* (Family: Fabaceae) roots, stem bark extracts of *Stereospermum Suaveolens* (Family: Bignoniaceae) *and Oroxylum indicum* (Family**:** Bignoniaceae), whole plants of *Bacopa monnieri* (Family: Plantaginaceae) *and Alysicarpus vaginalis* (Family: Fabaceae). Five different water extracts (600, 300. 150, 75, and 37.5 µg/ml) of each plant material were tested for human erythrocyte membrane stabilisation activity. The membrane stability activity of each plant material at different concentrations was measured spectrophotometrically at 540nm. Aspirin was used as the positive control and saline was used as the negative control. There was no *in vitro* human erythrocyte membrane stabilisation activity in the aqueous extracts of the plant materials used while aspirin at a concentration of 37.5µg/ml showed marked membrane stabilisation activity (25.45 ± 3.05 % inhibition). It is unlikely that anti inflammatory activities of *C.ternatea, O.indicum, S. Suaveolens, B.monnieri* and *A.vaginalis* are mediated via membrane stabilisation activity.

INTRODUCTION

In Ayurveda and traditional and folk medicine in several countries, decoction and extracts made from *Clitoria ternatea* (Family: Fabaceae) roots, stem bark extract of *Stereospermum Suaveolens* (Family: Bignoniaceae) *and Oroxylum indicum* **(**Family**:** Bignoniaceae), whole plant of *Bacopa monnieri* (Family:Plantaginaceae) *and Alysicarpus vaginalis*(Family: Fabaceae) are recommended to be used as treatments of several antimicrobial infections, anti fungal infections and to treat intra cellular parasitic infections (Ranaweera *et al.,* 2014, Tamaria *et al.,* 2013, Dinda *et al.,* 2015, Calabrese *et al.,* 2008, Sankaranarayanan 1988 and Facet *et al.,* 1999). At the same time these plants have been used to treat rheumatoid arthritic conditions, inflammation as well (Ranaweera *et al.,* 2014, Vanila *et al.,* 2008, Hosamini 2009 and Kumar *et al.,* 2007).

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Even though we have shown that *C.ternatea* roots has an antirhumatoid arthritic activity *in vitro* in one of our previous work (Ranaweera *et al.,* 2014), some of these ayurvedic and folk medicine claims have not been scientifically validated yet. Denaturation of proteins is one of the well documented causes of inflammation and arthritic disease (Umapathy *et al.,* 2010) and production of auto antigens in certain diseases could be due to the denaturation of proteins *in vivo* (Chandra *et al.,* 2012).Therefore it is fair to assume that some of these targeted proteins could be proteins found in plasma membranes of cells. If there are any natural product agents which have the ability in preventing or slowing down the denaturation of plasma membrane proteins, then these agents could be further tested for the production of novel anti- inflammatory drugs. The drugs called "Membrane stabilisers" are composed of cromones (sodium cromoglycate and nedocromil) and Ketotifen and inhibit the degranulation of mastocytes by a membrane stabilising effect (Mancel *et a.,* 1999).

Nowadays these are being used for prophylactic treatment of allergic asthma, rhinitis and allergic conjunctivitis and the manifestations of food allergy (Mancel *et al.,* 1999). Agents that can prevent protein denaturation through a membrane stabilisation mechanism therefore could be used further for novel anti-inflammatory drug development. Hence, Based on previous ayurvedic and folk medicine claims and also with our previous findings, we have designed an experiment to investigate the membrane stabilisation activity of water extracts of *C.ternatea* roots, *S.suaveolens and O.indicum* stem barks and whole plants of *B.monnieri*, *A.vaginalis*. In this study we have employed heat induced haemolysis of erythrocyte membrane was used due to its simplicity and reproducibility (Shinde *et al.,* 1999 and Ranasinghe *et al.,* 2012).

MATERIALS AND METHODS

Collection of plants materials

Mature plants of *C.ternatea* were uprooted from a home garden at Beliatta (geographical coordinates: 6°1'119" North, 80°45'2700" East situated in Hambantota district, Southern province of Sri Lanka in October 2014. The plants were identified and authenticated by Dr (Mrs) S. Ranwala, Department of Plant Sciences, University of Colombo Sri Lanka. A voucher specimen is deposited at Department of Medical Laboratory Science at General Sir John Kotelawala Defence University, Sri Lanka: (CR/01/2014). Dried pieces of the stem bark of *S.suaveolens and Oroxylum indicum* were purchased from the drug outlet of Wickramarachchi Ayurveda Institute in Gampaha District, Sri Lanka in October 2014. The stem bark has been identified by the pharmacognosist/purchasing officer of the drug outlet. Voucher specimens of *S.suaveolens* (SS/04/2014) and *Oroxylum indicum* (OI/05/2014) were deposited at the Department of Medical Laboratory Sciences at the Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Sri Lanka.

Whole plant of *Bacopa monnieri* and *Alysicarpus vaginalis* were collected in Beliatta (geographical coordinates: 6°1'119" North, 80°45'2700" East) situated in Hambantota district, Southern province of Sri Lanka, in October 2014. Whole plant of *Bacopa monnieri* and *Alysicarpus vaginalis* were identified and authenticated by Emeritus Professor (Mrs) A.S.Seneviratne , Department of Plant Sciences, University of Colombo, Sri Lanka. Voucher specimen of whole plants (BM/02/2014 , AV/03/2014) were deposited at the Department of Basic Sciences at the Faculty of Allied Health Sciences, General Sir John Kotelawla Defence University, Sri Lanka .

Preparation of aqueous water extracts of plant material

All plant materials were washed thoroughly in running tap water. Then the samples were air dried in a shade for 3-4 days until a constant weight was recorded. Then the plant materials were cut in to very small pieces and 60 g of individual plant material was boiled slowly in 1920 ml of distilled water for approximately for 3 hours until the final concentrated volumes of individual plant material was reduced to 240 ml.

The concentrated water extract solutions were filtered using a muslin cloth and the filtered waters extracts were freeze dried for further investigations.

Preparation of blood samples for membrane stabilization assays

Blood samples were collected from the investigators. Two millilitres of blood was freshly collected into K_3EDTA (F.L. Medical s.r.l. Torreglia, Italy) tubes. All the blood samples were stored at 4°C for 24 hours before use. An aliquot of 1.0 ml of blood got transferred into 1.5 ml micro-centrifuge tubes and was centrifuged at 2500 rpm for 5 minutes and the supernatant was removed. The cell suspension was washed with sterile saline solution (0.89% w/v NaCl) and centrifuged at 2500 rpm for 5 minutes. This was repeated three times till the supernatant was clear and colourless and the packed cell volume (PCV) was measured. The cellular component was reconstituted to a 40% suspension (v/v) with phosphate buffered saline (10 mM, pH 7.4) and was used in the assays.

Heat-induced hemolysis assay

The assay on inhibition of different plant extracts on heat-induced hemolysis of erythrocytes was carried out according to the method described by Okoli and the colleagues [15] with some modifications. Reaction volume of 1 ml, containing 30 µl of different concentrations of plant extracts (assay concentrations - 600, 300, 150, 75, 37.5 µg/ml) and 950 µl of saline (0.89 % w/v NaCl) were mixed and pre-incubated in a water bath (temperature was controlled by a thermostat with an accuracy of ± 0.1^{o}C; WiseBath, Daithan Scientific Co. Ltd, Seoul, Korea) at 55°C for 10 min. Then 20 µl of prepared erythrocytes suspension (40 %) was mixed and further incubated at 55°C for 20 min. After the incubation period samples were centrifuged at 5000 rpm at 4°C for 5 min. The absorbance of the supernatants was measure at 540 nm using a SPECTRAmax PLUS384™ microplate reader (Molecular Devices, Inc., CA, USA). Aspirin was used as the positive control and saline was used as the negative control. For sample blank incubations (absorbance produced by different plant extracts without heating) same procedure was carried out except incubating at 55 °C for 20 min. Inhibition of heat induced haemolysis of different plant extracts were calculated using following equation.

$$\text{Inhibition of haemolysis (\%)} = [(A_c - A_{nc}) - (A_s - A_{sb}) / (A_c - A_{nc})] \times 100$$

Where, A_c is the absorbance of the control, A_{nc} is the absorbance of negative control, A_s is the absorbance of samples and A_{sb} is the absorbance of sample blank.

Phytochemical screening

The water extracts of *C.ternatea*, *O.indicum* and *S. Suaveolens* were subjected to qualitative tests for flaonoids, polyphenols, tannings, alkaloids, steroids and terpenoids as described by Harry *et al.* (1996).

Statistical Analysis

Data presented as Mean ± Standard Error of Mean (SEM) and samples were used in quadruples (n=4). The values were calculated using Microsoft Excel 2007 package.

RESULTS

Effect of Aspirin (37.5µg/ml) on heat induced hemolysis of erythrocytes was 25.45 ± 3.05 % inhibition. Water extract of *C.ternatea* contained flavonoids, polphenols, tannings, alkaloid steroids, terpenoids and amino acids. Water extract of *S. Suaveolens* had unsaturated sterol and triterpenes, 2-deoxy sugars, flavonoids, tannins, saponins and primary and secondary alkaloids. *O.indicum* water extract contained primary, secondary, tertiary N function, quaternary amine base alkaloids, tannins and polyphenols of catechol type, unsaturated sterol and triterpenes.

DISCUSSION

Water extract of *C.ternatea* contained flavonoids, polphenols, tannings, alkaloid steroids, terpenoids and amino acids (Ranaweera *et al.,* 2014), while water extract of *S. Suaveolens* had unsaturated sterol and triterpenes, 2-deoxy sugars, flavonoids, tannins, saponins and primary and secondary alkaloids (Srivastava 2009). *O.indicum* water extract contained primary, secondary, tertiary N function, quaternary amine base alkaloids, tannins and polyphenols of catechol type, unsaturated sterol and triterpenes (Radhika *et al.,* 2011). This study evaluated *in vitro* membrane stabilizing potential of roots of *C.ternatea*, stem barks of *O.indicum*, *S.suaveolens* and whole plants of *B.monnieri* and *A.vaginalis* using aqueous extracts and head induced human erythrocytic haemolysis test.
Water was selected for extraction as many Ayurvedic, traditional and folk medicines are usually made in water. The bio assay used for the experiment was a well validated, sensitive, reliable and widely used in evaluation of membrane stabilisation of natural pharmacophores. The results obtained are summarised in Tables 1, 2, 3, 4 and 5. As shown, none of the waters extracts, at the concentrations tested possessed any erythrocyte membrane stabilising activity. Since erythrocyte membrane is reported to be similar to lysosomal membrane (Fong *et al.,* 1973). These results also indicate that these extracts may not induce any membrane stabilising effect on lysosomal membrane as well. Unfortunately, due to the interferences it was not possible to increase the concentrations of the extracts further.

Table 1. Effect of *C.ternatea* extract on heat induced hemolysis on erythrocytes.

Concentration (µg/ml)	% Inhibition
600	-36.69 ± 2.03
300	-65. 47 ± 4.91
150	-49.03 ± 3.99
75	-36. 74 ± 2.24
37.5	-19. 92 ± 3.94

(Data presented as Mean ± SEM, n=4)

Table 2. Effect of *A.vaginalis* extract on heat induced hemolysis on erythrocytes

Concentration (µg/ml)	% Inhibition
600	-34.91± 4.61
300	-40. 87 ±0.61
150	-39. 20 ± 3.08
75	-23. 39 ± 3.93
37.5	-16. 06 ± 1.76

(Data presented as Mean ± SEM, n=4)

Table 3. Effect of B. *monnieri* extract on heat induced hemolysis on erythrocytes

Concentration (µg/ml)	% Inhibition
600	5.58 ± 2.29
300	-8.26 ± 3.18
150	-5.35 ± 3.02
75	-1.93 ±6.23
37.5	1.02 ± 4.22

(Data presented as Mean ± SEM, n=4)

Table 4. Effect of *S. suaveolens* extract on heat induced hemolysis on erythrocytes

Concentration (µg/ml)	% Inhibition
600	-10.78 ± 3.97
300	-5.02 ± 8.38
150	-20.19 ±1.76
75	-6. 25 ± 5.03
37.5	-5. 07 ± 8.21

(Data presented as Mean ± SEM, n=4)

Table 5. Effect of *O.indicum* extract on heat induced hemolysis on erythrocytes

Concentration (µg/ml)	% Inhibition
600	2.37 ± 0.73
300	-5.77 ± 1.75
150	-7.55 ± 4.11
75	-13.65 ± 4.71
37.5	-7.11 ± 6.99

(Data presented as Mean ± SEM, n=4)

In contrast, the reference drug, aspirin at a concentration of 37.5µg/ml showed marked membrane stabilisation activity (25.45 ± 3.05 % inhibition). This is a novel but unexpected result since these plant materials are claimed to possess anti-inflammatory properties (Ranaweera *et al.,* 2014, Tamaria *et al.,* 2013, Dinda *et al.,* 2015, Calabrese *et al.,* 2008, Sankaranarayanan 1988 and Facet *et al.,* 1999) and according to Anosike *et al.* 2012, anti-inflammatory drugs usually offer protection of erythrocyte membrane against lysis induced by heat. This result may indicate that the observed anti-inflammatory activities of these plants are not related to their membrane stabilisation activity. Nevertheless saponins, flavonoids and triterpenoids are shown to possess membrane stability effects (Oyedapo *et al.,* 2010). Lack of membrane stabilising activity in our extracts may be due to masking action by other Phytoconstituents present in the extracts investigated. Further experiments are needed to support this notion.

Conclusion

Based on our experimental protocol and results, it is concluded that the claimed anti inflammatory activities of *C.ternatea, O.indicum, S. Suaveolens, B.monnieri* and *A.vaginalis* are unlikely to be mediated in a membrane stabilisation activity.

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