Phytochemistry and Immunopharmacological Investigation of *Rubia cordifolia* Linn. (Rubiaceae)

Marikani Kannan[a*], Ranjit Singh[b] and Narayanan[c]

a. P.G. Department of Microbiology, V.H.N.S.N.College, Virudhunagar-626 001, India.
b. Department of Advanced Zoology and Bio-technology, Sri Paramakalyani College, Alwarkurichi– 627 412, India.
c. Aquatic Biodiversity Center, St. Xavier's college, Palayamkottai - 627 002, India

Summary

Rubia cordifolia (Rubiaceae) is one of the important plants used by the Kani Tribes of Western ghats, Tirunelveli range, TamilNadu, India. The present investigation was carried out to find the phytocompounds and immunopharmacological properties of *Rubia cordifolia*. The ethanolic extracts of the whole plant was tested for many immuno-potentiating activity using a murine model. The active compound present in the extract enhanced both cell mediated and humoral immunity. Administering the extracts to rats that were given immunosuppressive drug, phosphamidon showed significant rejuvenation in immunity.

Keywords Immunity, phytomedicin, Immunopharmacology, *Rubia cordifolia*, macrophage, anti-inflammation, immunomodulation, hemagglutination.

*Corresponding author
E.mail microkannan@gmail.com

Introduction

The concept of modulation of immune responses to alleviate the diseases has existed in ancient systems of medicines like Ayurveda (Indian system of medicine) and Unani-Tib (Greco-Arab system of medicine). Plants had been extensively used to promote health and to maintain body's resistance against infection by potentiating immunity, re-establishing body equilibrium and conditioning of the body tissues[1, 2]. The modern system of medicine had always been enthusiastic to evoke non-specific defense mechanisms of human physiology, which led to the discovery of active immunization using microbial preparations to enhance the host defense against infection. Recently, the same enthusiasm has taken an important leap towards exploring a novel group of substances from natural resources that modulate the immune response of living systems[3].

The family Rubiaceae comprises about 450 genera and 6500 species and includes trees, shrubs and infrequently herbs. *Rubia cordifolia* is a perennial, herbaceous climbing plant, with very long roots, cylindrical, flexuous, with a thin red bark. Stems often have a long, rough, grooved, woody base. Anthraquinones a chief phytochemical present in many species of the Rubiaceae family exhibit antimicrobial, antifungal, hypotensive, analgesic, antimalarial, antioxidant, antileukemic and mutagenic functions [4, 5]. *Rubia cordifolia* is used to treat skin infection and also used as natural food colourants and as natural hair dyes [6].

Although *Rubia cordifolia* is used by traditional healers for many ancients there is no scientific account to validate the immunity enhancing potency of this plant. Hence in the present study attempt has been made to trace the influence of the extracts of *Rubia cordifolia* on humoral and cell mediated immune response in murine models.

Materials and Methods

Collection of plant materials

Healthy plant leaves were collected from Western Ghats of Tirunelveli range, Tamil Nadu, India. They were collected in early morning and were washed in tap water. They were shade dried for 10 days and powdered mechanically. The collected plant materials were botanically authenticated by the Botany Department, V.H.N.S.N.College, Virudhunagar, India.

Preparation of plant extract

Plant extracts were prepared by the method [7] , with slight modification to purify the ethanolic extract. Briefly, the extract was filtered through Whatman filter paper no. 1 to remove all unextractable matter, including cellular materials and other constituents that are insoluble in the extraction solvent. To obtain a concentrated crude ethanolic extract, the crude extracts were evaporated at 45^{o}C. The quantity was determined by weighing.

Phytochemical screening

Preliminary phytochemical screening tests were carried to find out the presence or absence of components like saponins, tannins, alkaloids, glycosides, flavonoids and essential oils [8].

Experimental animals

Swiss albino rats weighing 100 – 125 g of either sex were used to study the immunopharmacological activity. Rats were kept in 12 h light/12 h dark cycles under standard conditions of temperature (28^{o}C) and relative humidity (RH: 60%) with free access to food and water. All protocols performed in this study were conducted in accordance with internationally accepted principles for use and care of laboratory animals.

Experimental design

In each experiment, the animals were randomly divided into four groups and each group consisted of six animals. The animals in Group I served as a test control. Animals in Groups II & III were treated with the ethanolic extract of *Rubia cordifolia* 100 mg/kg, b.w. orally for 21 days, on 14th day group III received a single dose of cyclophosphamide 30 mg/ kg b.w. (i.p.) for inducing immunosuppression, and group IV received the same concentration of cyclophosphamide without plant extract treatment.

Preparation of sheep red blood cell (SRBC) antigen

Sheep blood was collected from a local slaughter house in sterilized container in the presence of an anticoagulant. SRBC were obtained by centrifugation and the cells were washed three times in Phosphate Buffer Saline (PBS) (pH 7.8). The SRBC antigen was prepared in PBS at a dose level of 1x10^{8} cells/ml.

Hematological changes

Group II and III animals, received *Rubia cordifolia* extract orally at a dose of 100 mg/kg for 21 days. On the 21st day, blood samples were collected from the orbital plexuses of individual animals and total WBC and RBC counts were determined with a haemocytometer (ROHAM, India).

Humoral immune response

Animals were divided into four groups as described above. Animals belonged to all groups were challenged with 0.2 ml of 10% sheep red blood cells (SRBC), i.p. on the tenth day after the starting of the experiment. Humoral immunity was studied in the experimental animals using the following assays.

Hemagglutination titer assay

Blood samples were collected from the orbital plexuses of individual animals on day 21 and the antibody titres were determined. Briefly, an aliquot (25 µl) of twofold diluted sera in isotonic saline was challenged with 25 µl of a 0.1% (v/v) SRBC suspension in microtitre plates. The plates were incubated at 37^{o}C for 1 h and then observed for haemagglutination. The antibody titres were expressed in a graded manner, the minimum dilution (1/2) being ranked as 1. The mean ranks of different groups were statistically compared.

Delayed-type hypersensitivity (DTH) reactions

In DTH studies the rats were primed with 0.1 ml of the SRBC suspension containing $1x10^{8}$ cells, i.p., on day 14 and challenged on day 21 with 0.05 ml of $2x10^{8}$ SRBC in the right hind foot pad. The control left hind paw received an equal volume of saline. The thickness of the foot pad (mm) was measured at 0, 12, 24, 36, and 48 h after challenge. The difference in the thickness of the right hind paw and the left hind paw was used as a measure of delayed-type hypersensitivity reaction [9].

Blood clotting time assay

A modified method of Lee and White[10] was used for the assay of blood clotting time. Using phosphate buffered saline (PBS) The plant extract was made up to different concentrations (0.1–1 ml). It was equilibrated in a water bath at 37^{0}C. Blood was collected from healthy adult volunteers by venipuncture into sterile plastic disposable syringes. The blood donors had been screened for the study by a medical practitioner to

ensure that they had not taken any medications for at least 1 week before the blood was collected. Starting a stopwatch, 1ml of this blood was immediately transferred into each of the equilibrated test tubes by carefully allowing the blood to run down the side of the tube. At intervals of 30 seconds, the tubes, still in the water bath, were gently tilted to an angle of 45^0C to check for blood clot formation. This was continued until the tubes could be inverted without blood flowing; the stopwatch was immediately stopped and the blood clotting time was recorded.

Statistical analysis

Statistical significance between the groups was analyzed using Student's t-test.

Results

Phytochemical screening

Preliminary phytochemical studies showed positive for alkaloids, cardiac glycosides, tannins, flavonoids and phenols, and negative for saponins, volatile oils, anthraquinones and cyanogenic glycosides, and very trace for steroids.

Hematological changes

Total white blood cells and red blood cells in the control and plant extracts treated rats are given in table 1. In the control rat the mean total WBC count was 13.08 ± 4.62 x 10^3/ µl. In *Rubia cordifolia* treated rats mean WBC count was 13.98 ± 2.68 x 10^3/µl. When the rats were given immune suppressive drug cyclophosphamide, the total WBC count got decreased (4.8 ± 1.3 x 10^3/ µl). However when the plant extract was given along with cyclophosphamide drug the decrement in total WBC was less when compared to the treatment in which cyclophosphamide alone was given. The results indicate that the active compounds in the extract of the plant had enhanced haematopoiesis.

Hemagglutination titer (HT) assay

In cyclophosphamide treated group, the antibody titre level decreased by 22.11 percent (Table 2). But In the rats that were given *Rubia cordifolia (*100 mg/kg), the antibody titre level increased by 14.74 percent. However in the group III treated with both immunosuppressant and plant extracts no significant changes was observed in humoral immune response.

Delayed-type hypersensitivity (DTH) reactions

Plants extract had significantly modulated DTH responses to SRBC (Fig 1). At 24h of injection, maximum enhancement of DTH response to SRBC was observed with *Rubia cordifolia* extract treated group (0.125±0.031, (138.89 %) N = 6, P<0.01) and the extract and cyclophosphamide treated group (0.102±0.043, (113.33 %) N = 6, P< 0.01). but the group of animals were treated with cyclophosphamide alone showed less DTH response (0.093±0.024, (13.33) N = 6, P< 0.01) when compared with control.

Assay of blood clotting time

The blood coagulation time of 4 adult volunteers varied between 4 and 8 min, with a mean of *6.*83±1.95 min. In the presence of 1ml of *Rubia cordifolia*, the mean of clotting time was significantly (*P* < 0.01) reduced to 2.83±1.01 min with a range of 2–4 min. The use of 1ml PBS instead of *Rubia cordifolia*, as a control, gave a mean clotting time of 7.29 ±1.98 min with a range of 5–9 min. In the tubes containing anticoagulant, the blood sample did not clot, as expected; this was a control used to ensure the integrity of the blood samples.

Table 1 Effect of ethanolic extract of *R.cordifolia* on peripheral blood count

Group	Treatment	Dose mg/kg	WBC X 10^3/µl	RBC X 10^6/ µl
I	Control (sterile water)	-	13.08 ± 4.62	10.73±1.34
II	*R. cardifolia* Linn. extract	100	13.98 ± 2.65* (6.3)	11.23±1.04* (4.7)
III	*R. cardifolia* Linn. extract (100 mg/kg) and cyclophosphamide (30 mg/kg)	130	5.97 ± 2.1 (-50.01)	9.73±0.04 (-9.3)
IV	cyclophosphamide	30	4.8 ± 1.3 (-63.9)	9.93±0.84 (-7.5)

Values are mean ± S.D. n of six rats. (Percentage change in parenthesis)

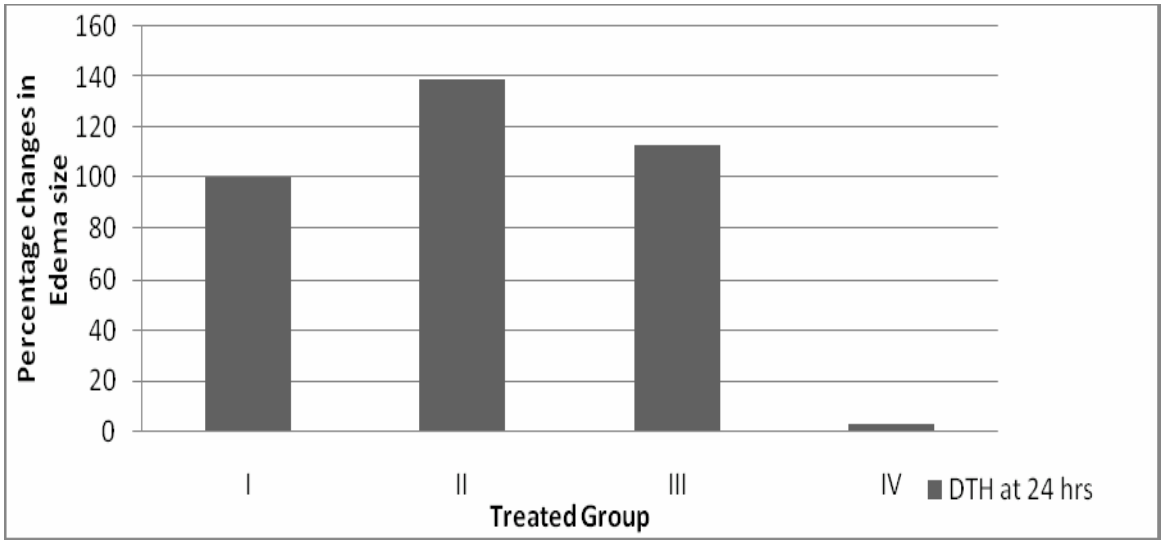
P<0.05 when compared with control.

Table 2 Changes in antibody titer in control, immunosupressed and ethanolic extract of *R.cordifolia* administrated rats

Group	Treatment	Dose mg/kg	Antibody titer
I	Control (sterile water)	-	4.07±0.03
II	*R. cardifolia* Linn. extract	100	5.03±0.05 (23.58)
III	*R. cardifolia* Linn. extract (100 mg/kg) and cyclophosphamide (30 mg/kg)	130	4.82±0.02 (18.43)
IV	cyclophosphamide	30	3.17±0.05 (-22.11)

Values are mean ± S.D. n of six rats. (Percentage change in parenthesis)
P<0.05 when compared with control.

Fig 1 Percentage changes in the edematous state of rat as a part of DTH response due to the treatment of ethanolic extract of *R.cordifolia*



I - Control (sterile water), II - *Rubia cordifolia,* Linn. extract treated, III - *R. cardifolia* Linn. extract and cyclophosphamide treated, IV- cyclophosphamide treated

Discussion

A preliminary phytochemical screening indicated the presence of compounds like alkaloids, cardiac glycosides, tannins, flavonoids and phenols were identified. The similar finding had also reported that alkaloids are prime phytochemical constituents in *Rubia cordifolia* [11]. *A*lkaloids had been reported to have pharmacological properties like antifungal activity [12], antiprotozoal, antimicrobial and antimalarial activities [13].

Oral administration of the extracts of *Rubia cordifolia* in rats had increased the total counts of WBC and RBC. It reflects that the stimulation of the haemopoetic system. *Rubia cordifolia* extract was found to increase the circulating antibody titre and antibody-forming cells. According to the literature the enhanced responsiveness of macrophages and subsets of T and B lymphocyte to plant extracts enhance the antibody synthesis[14].

DTH response in control and plant extracts treated rats showed significant differences. The plant extract treatment had significantly modulated DTH responses in SRBC treated rats. However when large doses of plant extract were given, DTH response gradually reduced with time. The suppressing DTH responses to SRBC by larger dose had been reported earlier [15] .

The paw edema in the right paw (receiving normal saline as control) and left paw edema (receiving SRBC) were compared and a significant ($P<0.05$) variation was noted in plant extract treated groups. However in animals treated with cyclophosphamide and plant extracts, a significant suppression of DTH was noticed. It was observed that the extracts, of the plant acted as a potentiator of DTH.

Blood clotting process is very complex, involving many factors found in the plasma and tissues. It involves both the intrinsic and extrinsic pathways [16, 17] (Brown, 1988; Jandl, 1996). Inhibitors (anticoagulants) and activators (procoagulant) of blood coagulation may affect many factors. The blood clotting test is used for distinguishing between the effects of *Rubia cordifolia* on the extrinsic and intrinsic pathways[18]. The extract of *Rubia cordifolia* had affected the extrinsic pathway factors: factors V, VII, X, prothrombin and fibrinogen as reported [19, 20, 21, 22].

The presence of alkaloids, cardiac glycosides, tannins, flavonoids and phenols in *Rubia cordifolia* were responsible for enhanced immunomodulation as reported earlier [23, 24]. Hence the extracts of the *Rubia cordifolia* offer a good shape for utilization to develop immunity enhancing drugs.

Acknowledgement

The authors would like to thank the co-ordinator, Department of Microbiology, V.H.N.S.N.College, Virudhunagar for providing facilities to carry out work and their kind assistants.

References

1. Savnur, HV., A Handbook of Ayurvedic Materia Medica with Principles of Pharmacology and Therapeutics. Dr. Jaghat, Belagaum, 1950,pp. xi–xxi.
2. Bhagwandash, V., Fundamentals of Ayurvedic Medicine. Bansal, Delhi, 1978, pp. ix–xvi.
3. Ranjith singh AJA., Dhasarathan, P., Sujatha N. and Jeypal. C., Antibacterial activities of *Cassytha capillaries*, Asian Jr. of microbial. Biotech. Env. Sc, 2004, 6 (4), 609 – 612.
4. Varier, P.S., Indian Medicinal Plants, A Compendium of 500 Species, vol. 4. Orient Longman, Hyderabad, India, 1995, pp. 269–272.
5. Jain, SK., DeFillips, RA., Medicinal Plants of India, vol. 2. Reference Publications, Algonac, 1991, MI, p. 558.
6. Chopra RN. Chopra IC, Handa KL, Kapur ID. *Indigenous Drugs of India*, UN Dhar and Sons Pvt. Ltd., Calcutta. 1992, 31 – 37.
7. Tiwari U, Rastogi B, Thakur S, Jain S, Jain NK, Saraf DK. Studies on the Immunomodulatory effects of Cleome viscose. Ind .J. Pharm. Sci. 2004. 66: 171-176.
8. Trease, G.E., Evans, M.S., Textbook of Pharmacognosy, 12. Bailliere Tindall, London, 1983, pp. 343–383.
9. Doherty NS. Selective effect of immunosuppressive agents against the delayed hypersensitivity response and humoral response to sheep red blood cells in mice. Agents Actions. 1981, 11: 237 - 242.
10. Wintrobe, M.W., Clinical Haematology, 6th ed. Lea and Febiger, Philadelphia, 1967, pp. 365–367.
11. Toshihiro, A., Gebreyesus, T., Hiroshi, H., Toshitake, T., Cooccurrence of C-24 epimeric 24-ethylsterols possessing and lacking a _25 bond in *Kalanchoe petitiana*. Phytochemistry. 1992, 31, 163–166.
12. Ali.Y., Munir. O ., Vahit B., Antioxidant activity of leaves *Cydonia vulgaris*. *J Agric Food Chem. 2001,* 29; 52(26):7884-90.
13. Frederich M, Jacquier MJ, Thepenier P, De Mol P, Tits M, Philippe G, Delaude C, Angenot L and Zaches Hanrot M,. Antiplasmodial Activity of Alkaloids from Various *Strychnos* Species *J. Nat. Prod*., 2002, 65, 1381.

14. Benacerraf. B., A hypothesis to relate the specificity of T lymphocytes and the activity of I region specific Ir genes in macrophages and B lymphocytes. J. Immunol. 1978, 120: 1809 -1812.
15. Sai Ram M, Sharma SK, Ilvazhagan G, Kumar D, Selvamurthy L., Immunomodulatory activity of polysaccharides from neem roots. *J. Ethnopharmacol*. 1997, 55: 133-139.
16. Brown, B.A., Haematology: Principles and Procedures, 5th ed. Lea and Febiger, Philadelphia, 1988, pp. 195–215.
17. Jandl, J.H., Blood: Textbook of Haematology, 2nd ed. Little, Brown & Co., Boston, 1996, pp. 1213–1275.
18. Descotes J. An Introduction to Immunotoxicology. Taylor & Francis, London. 1999, pp. 235- 238.
19. Agarwal SS and Singh VK ,. Immunomodulators: A review of studies of Indian medicinal plants and synthetic peptides: Part I Medicinal Plants, Proceedings of the *Indian National Science Academy*, 1999, 65;179-204.
20. Gupta, A.K., Chaugule, M.A., Pakdalkar, R.K., NMR structural characterization of gallactomannan from *Cassia occidentalis*. Indian Journal of Chemistry, 1995, 34, 169–170.
21. Kuby J. Immunology, Third ed. WH Freeman and Company, New York. 1997, pp.436- 439.
22. Elgert KD. Immunology: Understanding the Immune System.Wiley, New York. 1996, pp. 306-308.
23. Gutali K, Ray A, Debnath PK, Bhattacharya SK. Immunomodulatory activity of Melia azedarach aqueous leaf extracts on murine lymphocytes. J. Ind. Med. Plants. 2002, 2: 121-131.
24. Ranjith M.S., Ranjith singh A.J.A. and Gokulshanker, S., Enhanced phagocytosis and antibody production by *Tinospora cardifolia* – A new dimension in immunomodulation. African Jr. of. Biotechnology, 2008, 7 (2), 081 – 085.