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Evaluation of Immunomodulatory Activity of Infusion of Rourea Coccinea ((Schumach. &Thonn.) Benth) Root Bark in Wistar Rat

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Abstract: *Rourea coccinea ((Schumach. &Thonn.) Benth) has been described as one of medicinal plants used in the treatment of various diseases in West Africa. Recent studies have shown the antipyretic, anti-inflammatory and analgesic properties of hydro-ethanolic extract of the bark of its root. Could it have an effect on the immune system? The aim of this work was to study the immunomodulatory activity of the infusion of Rourea coccinearoot bark in Wistars rats. For this study, batches of three rats were formed. The immunomodulatory activity of the infusion at doses of 500, 1000 and 1500 mg.eq.mv/kg administered orally were evaluated in normal rats and immunosuppressed rats. Total white blood count and lymphocyte count, antibody titre, and delayed type hypersensitivity reaction were determined using standard methods and procedures. Statistical analysis was performed using GraphPad prism 5.00 Software. The results showed that infusion of Rourea coccinea bark would not cause a significant increase in the number of white blood cells or circulating lymphocytes in normal rats and does not induce restoration of the same parameters after immunosuppression induced by cyclophosphamide. It has on the other hand, a stimulatory effect of the production of antibodies at the dose of 500 mg.eq.mv/kg (effectors of the humoral mediated immune response). The infusion of the bark of root of Rourea coccinea therefore has a stimulatory effect of humoral immunity at the dose of 500 mg eq.mv/kg.*

Keywords: *Immunomodulation; Rourea coccinea; Wistar rat*

1. Introduction

The immune system protects the organism from pathogens and invaders that compromise its harmonious functioning. For its defence, the mechanisms put in place may be sometimes exaggerated, ineffective or insufficient, this encourages the installation of diseases. The increases in infectious, inflammatory, autoimmune, neoplastic diseases and bacterial resistance are all situations that put people at risk (Davis *et al*., 2000). The difficulties of access to conventional medical care on the one hand and the side effects (sometimes very harmful) of certain treatments on the other hand are major challenges facing medical biotechnology. Thus, the eye is increasingly turning to the search for medicinal plants that provide bioactive substances, which are accessible at low cost and have a favourable benefit-risk ratio (WHO, 2000).Several plants used in traditional medicine around the world have therefore been studied and some of them have revealed their ability to modulate the immune response. These include *Viscum album, Panax ginseng, Asparagus racemosus, Azadirachtaindica, Tinosporacordifolia, Polygala senega, Ocimumsantum* (SaiRam *et al*., 1997, Estrada *et al*., 2000, Mediratta *et al*., 2002). In West Africa, *Rourea coccinea* (Schumach. &Thonn) Benth) has been described as one of these medicinal plants used in the treatment of various diseases (Adjanohoun *et al*., 1986; Dalziel, 1937). Recent studies have demonstrated anti-inflammatory, analgesic, antidiarrhoeal and antipyretic activity (Akindele and Adeyemi, 2006 ab, 2007 ab, Dossehet *al*., 2014) of the leaves and bark of the root of this plant. This suggests that these different parts of the plant may have immunomodulatory properties. The roots of Rourea coccinea have proved to be practically non-toxic (Dosseh *et al*., 2015).Therefore in the present work infusion of *Rourea coccinea* root bark is evaluated for its immunomodulatory effect.

2. Materials and Methods

2.1. Plant Material Collection

Roots of *Rourea coccinea* were collected around the campus of "University of Lomé" in the month of February, 2013 and was identified and

authenticated at the Herbarium of Botany department of aforementioned University. The specimen is recorded under the number Togo 15075 and the name *Byrsocarpus coccineus*. The roots, collected the same day was cleaned, the bark was removed and made to dry under air-conditioning with the shelter of the sun during one week at the Laboratory of Physiology and Pharmacology of the Natural Substances of the Formation and Research Center on the Medicinal Plants (CERFOPLAM) of the University of Lomé, Togo. Dried material was pulverized to powdered with mill (Thomas Scientific Laboratory Mill Model 4, the USA).

2.2. Infusion Preparation

Hot distilled water was added to the powder (1:10 plant-water weight ratio). The mixture was filtered after cooling (using cotton),placed in a glass scintillation vial and stored at 4°C (Senchina *et al.*, 2005). Immunological tests were carried out with infusion at the various doses of 500; 1000 and 1500 mg eq.mv/kg.

2.3. Experimental Animals

Healthy young adult albino wistar rats (100 – 150g) of either sex were used. These animals, produced by the Department of Physiology/Pharmacology of ‘’Université de Lomé’’ were kept under natural environmental conditions with 12 h light/dark cycle and fed with diet(containing 72% corn, 8% fish meal, 10% sound of wheat and 10% soya) and water *ad libitum.*

2.4. Antigen Preparation

Fresh blood was collected from healthy sheep sacrificed in local slaughterhouse in Alsever’s solution (1:1) (Nfambi *et al.*, 2015). The blood was then centrifuged at 3000 rpmfor 5 min to enable red blood cells to settle at the bottom of the test tube. The supernatant was discarded, leaving sheep red blood cells (SRBCs) pellets that were washed four times in large volumes of physiological normal saline solution and adjusted to a concentration of $1x10^8$ cells/mL. The antigen solution thus prepared is kept carefully with 4C for immunization and challenge.

2.5. Drugs

Oncomide® (KHANDELWAL LABORATORIES Pvt. Limited, B-1, Wagle Industrial Estate, Thane - 400 604, Regh. Office: 79/87, D. Lad Path, Mumbai -400 033, India, Batch N PCYC41209; Expiry: 11/2017) a product containing of Cyclophosphamide (out of injectable powder IP - 500 Mg) was used as immunosuppressor.

2.6. Group Treatments

Rats were divided into four groups of three animals each. The infusion was administered orally for 14 days at doses of 500; 1000 and 1500 mg eq.mv/kg/day (Groups II – IV). Rats in the normal control group (Group I) received distilled water throughout the 2 weeks of the study period.

2.7. Effect of Infusion of Rourea Coccinea on Total Leukocyte Count and Lymphocytes Count

Animals were divided into four groups (I–IV). Each group comprised of three animals. Group I (control) received distilled water; group II, plant infusion 500mg eq.mv/kg body weight; group III, plant infusion 1000 mg eq.mv/kg; and group IV, plant infusion 1500 mg eq.mv/kg daily for 14 days. 24 hours after the last administration, blood was drawn by plexus retro-orbital into ethylenediaminetetraacetic acid (EDTA)-containing vacutainers. It was then analyzed at the National Institute of hygiene hematology laboratory using an hematology analyzer (Sysmex KX-21N Serial N° BO216) for the total leucocyte and lymphocyte counts(Singh *et al.*, 2011).

2.8. Effect of Infusion of Rourea Coccinea on Humoral Antibody Titer

On 0^{th} day, rats of all groups were sensitized by injecting 0.1 mL of solution of SRBCs containing 1×10^8 cells, intraperitoneally (i.p.). Animals were then divided into four groups (I – IV) each containing three animals and are subjected to the same treatment like previously for 14 days.24 hours after the last administration, blood was withdrawn from retro-orbital plexus of each animal. Blood samples were centrifuged at 3000 rpm and the serum separated. Antibody titers were then determined by the lysis of red blood cells technique. Serial twofold dilutions of serum were made with normal saline in micro-titer plates of 96-well (12x8 U bottomed). Wells were marked from I to XII and the dilution was carried out as follows

- 50 µL of solution normal saline (NaCl 9‰) were put in each well of micro-titer plate of 96 wells;
- 50 µL of serum is added to the contents of the 1^{st} well containing 50 µL of solution normal saline; 50 µL of diluted serum was added to

2nd well containing 50 µL normal saline. Such serial dilutions were done till 12thwell.

- 50 µL of solution of SRBCs (containing 1×10^8 cells/mL) is added to the contents of each well.

Microtitre plates were incubated at room temperature for three (3) hours (Davis and Al, 2000) and examined visually for hemolysis. It was a quantitative test and the serial dilution was made to determine the highest factor of dilution giving a positive test (lysis of the red blood cells). This factor of dilution corresponds to the antibody titer (HA units/µL).

2.9. Delayed Type Hypersensitivity (DTH)

Animals were sensitized by injecting 0.1 mL of SRBCs suspension, containing 1×10^8cells, intraperitoneally (i.p.), on day 0 and were divided into four groups (I – IV) each containing three animals. They were subjected to the same treatment like previously for 14 days. Two hours after the last administration, the thickness of the right hind footpad was measured using method of Agbonon *et al.*, (2001). After this, animals were challenged by injecting the same amount of SRBCs suspension into the right hind footpad. The thickness of the right hind footpad was measured 24 h after the challenge(Vogel, 2002). The difference of volume, before and 24h after the challenge is regarded as the response to the reaction of delayed over-sensitiveness (Davis *et al.*, 2000).

2.10. Drug Induce Immunosuppression

On 0th day, blood of rats was withdrawn from retro-orbital plexus to hematological parameter determination (primarily total leukocyte and lymphocytes). Rats were then divided into five groups each containing three animals. The infusion was administered orally for 13 days.

- Group I: distilled water 10 mL/kg b.w, + NaCl 9‰ at 11th, 12th, 13th day (i.p.)
- Group II: distilled water 10 mL/kg + Cyclophosphamide 30 mg/kg b.w at 11th, 12th, 13th day (i.p.)
- Group III: infusion of the bark of root of R. coccinea (500 mg éq.mv/kg) + Cyclophosphamide 30 mg/kg b.w at 11th, 12th, 13th day (i.p.)
- Group IV: infusion of the bark of the root of R. coccinea (1000 Mg éq.mv/kg)) + Cyclophosphamide 30 mg/kg b.w at 11th, 12th, 13th day (i.p.)
- Group V: infusion of the bark of the root of R. coccinea (1500 Mg éq.mv/kg)) + Cyclophosphamide 30 mg/kg b.w at 11th, 12th, 13th day (i.p.)

On day 11th, 12th and 13th, one hour after the last administration, cyclophosphamide30 mg/kg b.w was given to animals except control group. On day 14th, blood was withdrawn from retro- orbital plexus of animals of each group and subjected to hematological parameters determination and restoration of parameters were observed (Gaikwad *et al.*, 2011).

2.11. Statistical Analysis

All values were expressed as mean ± SEM. Results were analyzed statistically using One-way ANOVA followed by Tukey's multiple comparisons using the software GraphPad PRISM 5.00 (GraphPad Software Inc, CA, USA).The difference was considered significant if $p<0.05$.

3. RESULTS

3.1. Effect of Infusion of Rourea Coccinea on Total Leukocyte Count and Lymphocytes Count

Infusion of *Rourea coccinea* (500, 1000 and 1500 mg éq.mv/kg p.o.) was administrated for 14 days. The results obtained indicate that there was not a significant increase in mean of total leukocyte count and lymphocytes count when compared to control group (Tables 1; 2).

Table1. *Effect of infusion of Rourea coccinea on total leukocyte count*

Groups	Treatments	Doses (mg eq.mv /kg)	Leucocyte count ($\times10^3$/µL)	
			0th day	14th day
1	Distilled water	10 (a)	7,53± 0,80	8, 40 ± 0,35
2	IER *R. coccinea*	500	8,63± 1,20	10,60± 1,55
3	IER *R. coccinea*	1000	8,80± 1,08	10,70 ± 1,05
4	IER *R. coccinea*	1500	11,90 ± 1,63	15,83 ± 1,56

Values are expressed as Mean ± SEM, (n=3), p values as significant* if $p<0.05$compared to control group. (a) : mL/kg

Table2. *Effect of infusion of Rourea coccinea on total lymphocyte count*

Groups	Treatments	Doses (mg eq.mv /kg)	Lymphocyte count (×10³/µL)	
			0th day	14th day
1	Distilled water	10 (a)	6,12± 0,76	6,91 ± 0,84
2	IER*R. coccinea*	500	6,41± 0,48	8,23± 0,93
3	IER*R. coccinea*	1000	6,74± 0,45	7,95 ± 0,18
4	IER*R. coccinea*	1500	9,67± 0,81	12,29 ± 0,02

Values are expressed as Mean ± SEM, (n=3), p values as significant* if p<0.05 compared to control group. (a) : mL/kg

3.2. Effect of Infusion of Rourea Coccinea on Humoral Antibody Titer

Oral administration of *Rourea coccinea* infusion (500, 1000 and 1500 mg éq.mv/kg p.o.) for 14 days showed the following reaction in rats. Compared to the control group, the results obtained indicate that animals treated with lower dose (500 mg éq.mv/kg) showed significant increase humoral antibody titer (Table 3).

Table3. *Effect of infusion of Rourea coccinea on Humoral Antibody Titer*

Groups	Treatments	Doses (mg eq.mv/kg)	Antibodytiter (unités/µL)
1	Distilled water	10 (a)	10,67 ± 2,66
2	IER *R. coccinea*	500	**42,67 ± 10,67***
3	IER *R. coccinea*	1000	26,67 ± 5,33
4	IER *R. coccinea*	1500	26,67 ± 5,33

Values are expressed as Mean ± SEM, (n=3), p values as significant* if p<0.05 compared to control group. (a) : mL/kg

3.3. Delayed Type Hypersensitivity

The result indicates that there was not significant (p<0.01) increase in paw thickness of treated groups 24 hours after the challenge when compared with control group (Table 4).

Table4. *Effect of infusion of Rourea coccinea on Delayed Type Hypersensitivity*

Groups	Treatments	Doses (mg eq.mv/kg)	DHT (mL)
1	Distilled water	10 (a)	1,18 ± 0,01
2	IER *R. coccinea*	500	0,90 ± 0,1
3	IER *R. coccinea*	1000	1,15 ± 0,10
4	ER *R. coccinea*	1500	1,25 ± 0,07

Values are expressed as Mean ± SEM, (n=3), p values as significant* if p<0.05 compared to control group. (a): mL/kg

3.4. Drug Induced Immunosuppression

Cyclophosphamide treatment for the period of 3 days showed significant reduction in white blood cells count and lymphocyte count (p<0.05) and thereby exerted an immunosuppressant effect when compared to control group. Combined treatment of *Rourea coccinea* infusion and immunosuppressive drug at all doses not showed restoration of white blood cells count and lymphocytes count (p<0.05) when compared cyclophosphamide groups (Table 5, 6).

Table5. *Effect of infusion of Rourea coccinea on total leukocyte count after immunosuppression*

Groups	Treatments	Doses (mg eq.mv/kg)	Leucocyte count (×10³/µL)	
			0th day	14th day
1	Distilled water + NaCl (9‰)	10 (a)	9,33 ±0,85	9,93± 0,88
2	Distilled water + Cyclophosphamide)	10 (a)	7,73± 0,83	1,07± 0,14
3	IER *R. coccinea* + Cyclophosphamide	500	9,16± 0,40	2,50± 0,55
4	IER *R. coccinea* + Cyclophosphamide	1000	8,66± 0,69	1,75± 0,16
5	IER *R. coccinea* + Cyclophosphamide	1500	12,56 ±0,43	2,80± 0,47

Values are expressed as Mean ± SEM, (n=3), p values as significant* if p<0.05 compared to control group. (a) : mL/kg

Table6. *Effect of infusion of Rourea coccinea on total lymphocyte count after immunosuppression*

Groups	Treatments	Doses (mg eq.mv /kg)	Lymphocyte count ($\times 10^3/\mu L$)	
			0^{th} day	14^{th} day
1	Distilled water + NaCl (9‰)	10 (a)	7,75 ±0,73	8,24± 0,76
2	Distilled water + Cyclophos phamide	30 (a)	6,3 ±0,73	0,91± 0,16
3	IER *R. coccinea* + Cyclophos phamide	500	8,40 ± 0,46	2,00± 0,56
4	IER *R. coccinea* + Cyclophos phamide	1000	7,68 ± 0,74	1,52± 0,17
5	IER *R. coccinea* + Cyclophos phamide	1500	11,53 ± 0,52	2,21± 0,36

Values are expressed as Mean ± SEM, (n=3), p values as significant* if p<0.05 compared to control group. (a) : mL/kg

4. DISCUSSION

The immune system is the defense mechanism of the body. Modulation of the immune system helps in maintaining a disease-free state within an individual. Immunomodulators have therefore been used globally to control disease conditions (Nfambi *et al*., 2005). In the present study, the immunomodulatory activity of root bark of *Rourea coccinea* by evaluating its effect ontotal leukocyte count and lymphocyte count, Delayed Type Hypersensitivity (DTH), Humoral Antibody titre (HA) and immunorestoration test using wistars rats.

Oral administration of infusion at different doses (500, 1000 and 1500 mg eq.mv/kg) during 14 days showed no significant increase in total leukocyte count and lymphocyte count when compared with the control group. Similar results were obtained by Dosseh *et al*. (2015) who administered ethanolic extract *Rourea coccinea* root bark(400 and 800 mg / kg, p.o) for four weeks showed no significant increase of the same parameters when compared to the control. Infusion of *Rourea coccinea* root bark therefore does not lead to increase the number of immune cells.

The DTH reaction is a type IV cell-mediated immune response according to the Coombs and G ell classification of hypersensitivity reactions, 1975(Rajan, 2003). DTH reaction is antigen specific and provides functional assessment of cell-mediated immunity. It cause erythematic or edema at the site of antigen injection in immunized animals when encountered with activated Th1 (T-helper cells) cells. DTH comprises of two phases, an initial sensitization phase and effector phase. In initial sensitization phase Th1 cells are activated and clonally expanded (Anarthe *et al*., 2014). In effector phase subsequent exposure to the antigen induces DTH response where activation of the T cells leads to the release of lymphokines which causes the activation and accumulation of macrophages, increases vascular permeability, induces vasodilatation and produces inflammation (Janeway, 2001).This results in the net increase in the thickness of the foot pad which is visible only after 16 to 24 hours after the challenge(Vogel, 2002). In the present study, Sheep Red Blood Cells (SRBCs) were used as antigens. Oral administration of infusion at different doses (500, 1000 and 1500 mg eq.mv/kg) during 14 days did not show any significant increase in paw edema as compared with the control.This result indicates that infusion of *Rourea coccinea* root bark has no effect on the initial sensitization phase (clonally expanded of cells), the effector phase (release of lymphokines) or on the accessory cell typesrequired for the expression of the reaction.

The antibody titer test was performed to determine the effect of the infusion of *Rourea coccinea* root bark on the humoral immune response. Humoral immunity involves interaction or co-operation between T and B-lymphocytes, macrophages and B cells with the antigen then their subsequent proliferation and differentiation into plasma cells that secretes antibodies. Antibodies thus function as the effectors of the humoral response by binding to the antigens and neutralizing them or facilitating their elimination (Sudha*et al*., 2010). The antigen-antibody complexes thus formed react

with a system of serum proteins (the complement system).If this reaction occurs on a cell surface, the result will be the formation of transmembrane pores and destruction of the cell. Activation of the complement system will lead to lysis of SRBCs used as antigens (Goldsby *et al.*, 2003). The presence of antibodies in the serum results in the "pink" coloring of the solution contained in the wells (of the microplate), characteristic of the hemoglobin released during the lysis of SRBCs. Oral administration of infusion of *Rourea coccinea* root bark (500, 1000 and 1500 mg eq.mv/kg) for 14 days indicated that only animals treated with lower dose (500 mg eq.mv/kg) showed a significant increase humoral antibody titer when compared with the control group. At lower dose (500 mg eq.mv/kg), infusion of R*ourea coccinea* root bark would therefore stimulate the humoral mediated immune response.

Cyclophosphamide is a very powerful cytotoxic and immunosuppressive agent that acts at different levels on cells involved, in the defense mechanisms against various invaders by inhibiting both cell-mediated immunity and humoral immunity. It also causes myelosuppression (dose-dependent) and a significant reduction in hemoglobin, the number of red blood cells and circulating white blood cells (Cancer, 1975). Cyclophosphamide treatment (i.p.) for the period of 3 days showed significant reduction in total leukocyte count (1.07 ± 0.14, $p < 0.01$) and lymphocytes count (0.91 ± 0.16, $p < 0$, 01) when compared with the control group (NaCl, i.p.). Cyclophosphamide had an immunosuppressive effect in rats. Combined treatment of infusion and immunosuppressive drug at all doses of infusion not showed restoration of the same parameters when compared to cyclophosphamide only treated groups. Oral administration of infusion of *Rourea coccinea* root bark could not restore the circulating immune cells after immunosuppression induced by the cyclophosphamide.

5. Conclusion

Based on the results obtained in the present study, infusion of *Rourea coccinea* root barkhas the potential to stimulate at lower dose humoral immune responses (antibody titer) in wistar rats but it does not lead to increase the number of immune circulating cells, has no effect on cell-mediated immune and cannot induce restoration of the circulating immune cells after immunosuppression induced by cyclophosphamide. However, further studies would be needed to evaluate the effect infusion of *Rourea coccinea* root bark at doses below 500 mg eq.mv / kg on humoral immunity in combination with other antigens to confirm the effect immunostimulatory effect and the immuno-adjuvant effect of this infusion. The use of other study methods and the exploration of more specific immunity parameters would also be necessary to confirm the immunostimulatory effect observed.

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