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EFFECT OF *PUTRANJIVA ROXBURGHII* WALL. ON PHAGOCYTOSIS AND CHEMOTAXIS BY POLYMORPHONUCLEAR LEUKOCYTE CELLS (*IN-VITRO* STUDY)

Madhavi Badole

Department of Chemistry, Ramnarain Ruia Autonomous College, Matunga Mumbai - 400019, Maharashtra, India.

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Correspondence to Author:
Dr. Madhavi Badole

Department of Chemistry, Ramnarain Ruia Autonomous College, Matunga Mumbai - 400019, Maharashtra, India.

E-mail: madhavibadole@gmail.com

ABSTRACT: Polymorphonuclear leukocytes or neutrophils are important components of host defense machinery. They are the first line of defense against invading microorganisms. In the present research work, ability of human neutrophils to phagocytose was studied *in-vitro* by exposing the polymorphonuclear leukocytes to *Candida albicans* and the phagocytic capacity was evaluated using aqueous extract of leaves powder of *Putranjiva roxburghii* Wall. The chemotactic activity that is the movement of polymorphonuclear leukocytes towards a chemical stimulant was studied *in-vitro* by placing polymorphonuclear leukocytes in presence of a chemotactic agent Zymosan using aqueous extract of leaves powder of *Putranjiva roxburghii* Wall.

INTRODUCTION: The immunomodulatory activity of *Tinospora cordiflia* Mires. (Rasayana plant) has been reported in literature. The percent phagocytosis and phagocytic index of aqueous extract of *Tinospora cordiflia* Mires. has been studied *in-vitro* and was found to stimulate poly-morphonuclear (PMN) cells isolated from normal healthy volunteers [1]. However, the immuno-modulatory activity of *Putranjiva roxburghii* Wall. has not been reported in literature. *Putranjiva roxburghii* Wall. has been described in literature [2] as a 'Rasayana' plant and most of the Rasayana plants like *Tinospora cordifolia* Miers., *Emblica officinalis* Gaertn., *Asparagus racemosus* Willd. exhibit immunomodulatory activity and increase the body immunity against diseases [2].

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Therefore, in the present research work efforts has been made to evaluate the immunomodulatory activity of *Putranjiva roxburghii* Wall.

EXPERIMENTAL: *Putranjiva roxburghii* Wall. leaves were collected from 'Keshav Shrushti', Mumbai, India and authenticated from Botanical Survey of India, Pune. (Voucher no. BSI/WC/Tech/2008/177). The leaves were washed with water to remove soil particles, dried in the shade, and finely powered. The powder was passed through the 85 mesh sieve and stored in an airtight container at room temperature (28º ± 2 ºC).

Preparation of Extract of *Putranjiva roxburghii* Wall: 10.0 g accurately weighed leaf powder of *Putranjiva roxburghii* Wall. was extracted using 100.0 cm^3 of methanol in a Soxhlet apparatus consecutively for 8 days, till the methanol in the soxhlet apparatus was colourless. The extract was then filtered and concentrated at 57 ºC in a water bath till the dry powder was obtained.

Preparation of Working Concentrations of *Putranjiva roxburghii* Wall. (50-800 µg/cm^3): 50

µL, 100 µL, 200 µL, 400 µL, 600 µL and 800 µL aqueous extracts of *Putranjiva roxburghii* Wall. (1000 µg/cm^3) were transferred to six different vials separately. To this extract of *Putranjiva roxburghii* Wall. 950 µL, 900 µL, 800 µL, 600 µL, 400 µL, and 200 µL of Minimum Essential Medium were added separately to get the working concentrations of *Putranjiva roxburghii* Wall. (50-800 µg/cm^3).

Positive Control: The aqueous extract of *Tinospora cordifolia* Miers., a known plant immunostimulant in the concentration of 400 µg/cm^3 was used as control [2].

Preparation of *Tinospora cordifolia* Miers. Extract (400 µg/cm^3): About 8.0 mg of methanol extracted stem powder of *Tinospora cordifolia* Miers. was weighed accurately and dissolved in 20.0 cm^3 sterile distilled water.

Ethics Committee Permisssion: The protocol was submitted to the Committee for Academic Research Ethics, Seth G.S. Medical College and KEM hospital, Parel, Mumbai, to carry out in-*vitro* study using leaf extract of *Putranjiva roxburghii* Wall. and a written consent was obtained to carry out *in vitro* study on human subjects before initiation of study.

Isolation of Polymorphonuclear Leukocyte:
Method: After taking written, valid and informed consent, six normal healthy male volunteers were recruited in the study. 24.0 cm^3 of peripheral venous blood was collected from each volunteer in a sterile heparinised tube and polymorphonuclear leukocytes were separated.

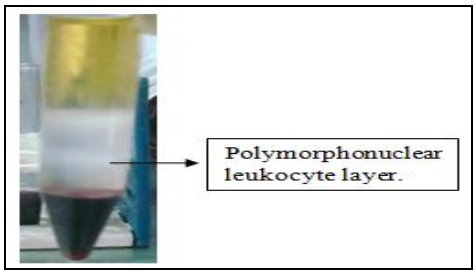

FIG. 1: THE POLYMORPHONUCLEAR CELLS BETWEEN TWO LAYERS OF HISTOPAQUE

The polymorphonuclear leukocytes obtained by the above method were adjusted to 2 × 10^6 cells/cm^3 for phagocytosis and 2.5 × 10^6 cells/cm^3 for chemotaxis.

Preparation of *Candida albicans* Suspension (1.0 × 10^{6}cells/cm^3): *Candida albicans* was maintained on Sabouraud's agar. A loopful of the culture was inoculated in 2.0 cm^3 of Sabouraud's agar 18 h prior to the experiment at room temperature to allow it to enter yeast phase.

After 18 h, a loopful of culture was transferred to about 2.0 cm^3 of 0.9% saline. 20µL of culture was mixed with 380µL of normal saline. Viability of polymorphonuclear leukocyte was assessed using Trypan dye exclusion method [3]. 20 µL of polymorphonuclear leukocyte was mixed with 20 µL of 0.1% Trypan blue dye. This mixture was then mounted on Neubaur chamber and the number of cells was counted. The count was adjusted to1.0 x 10^6 cells/cm^3 with Minimum Essential Media as follows.

The cell count of *Candida albicans* in one square of Neubaur chamber = 78

Total number of *Candida albicans* present in 400 µL suspension was = 15.6 × 10^6 cells/cm^3

The above count of *Candida albicans* was adjusted to 1.0 × 10^6 cells/cm^3 by mixing 1.0 cm^3 of *Candida albicans* culture with 14.6 cm^3 Minimum Essential Media. Similar procedure of cell count adjustment was followed for each of the volunteer.

Viability Assay for Polymorphonuclear Leukocyte: Viability of polymorphonuclear leukocyte was assessed by using Trypan dye exclusion method [4]. Percentage of unstained leukocytes reflects the percent population of the viable cells. Polymorphonuclear leukocytes with viability more than 90% were considered as non toxic and were selected for estimation of phagocytosis and chemotaxis. The percent viability of polymorphonuclear leukocytes of each of the volunteer was found out by above procedure.

The percent viability for each volunteer was calculated by using following formula and the percent viability obtained for each volunteer is given in **Table 1**.

$$\% \text{ Viability} = \frac{\text{Total number of cells} - \text{Total number of dead cells}}{\text{Total number of cells}} \times 100$$

TABLE 1: RESULT OF VIABILITY OF POLYMORPHONUCLEAR LEUKOCYTE CELLS ISOLATED FROM HEALTHY HUMAN VOLUNTEERS

Vol. no	Total number of cells present in one square of Neubaur chamber	Total number of dead cells	Percent viability
1	38	0	100
2	36	1	97.2
3	42	0	100
4	28	0	100
5	32	1	97.2
6	40	1	97.2

Since the percent viability of neutrophil suspension was more than 90%, the neutrophil suspension was used to carry out assay for phagocytosis and chemotaxis.

Assay for Phagocytosis: [4] To carry out the assay for phagocytosis eight test tubes were taken and were numbered from 1-8. 20 µL each of the concentration of *Putranjiva roxburghii* Wall. (50-800 µg/cm^3) was taken in a different test tube from 2 to 7 and 20 µL *Tinospora cordifolia* Miers. (400 µg/cm^3) was taken in test tube number 8. The assay system was prepared for each of the six volunteer as given in **Table 2**.

TABLE 2: ASSAY SYSTEM PREPARED FOR ESTIMATION OF PHAGOCYTOSIS

Test tube no.	Conc. of *P. roxburghii* added (µg/cm^3)	Conc. of *T. cordifolia* added (µg/cm^3)	Suspension of *C. albicans* added in µL (1.0 × 10^6 cells/cm^3)	Suspension of PMN leukocyte added in µL (2.0 × 10^6 cells/ cm^3)	Minimum essential media added in µL
1	--	-	250	250	500
2	50	-	250	250	500
3	100	-	250	250	500
4	200	-	250	250	500
5	400	-	250	250	500
6	600	-	250	250	500
7	800	-	250	250	500
8	-	400	250	250	500

Each of the above test tubes prepared for phagocytosis assay was incubated at 370 ºC in 5 percent CO_2 environment for 60 min. Each of the test tubes was centrifuged in a cytocentrifuge at 1500 rpm for 15 min. Polymorphonuclear leukocytes cells button was obtained in each of the test tube. The supernatant obtained was discarded. A smear of polymorphonuclear (PMN) leukocytes was prepared on a glass slide for each concentration of *Putranjiva roxburghii* Wall. and *Tinospora cordifolia* Miers. on the glass slide, and each slide was kept in cytospin bucket and the slides were cytospined at 1500 rpm for 15 min. The smear obtained on each of the slide was then stained by flooding the smear with freshly diluted Geimsa stain (1:8) for 30 min.

Each of the slide was washed gently under running tap water. Each slide was then examined for phagocytosis by light microscopy under oil immersion lens.

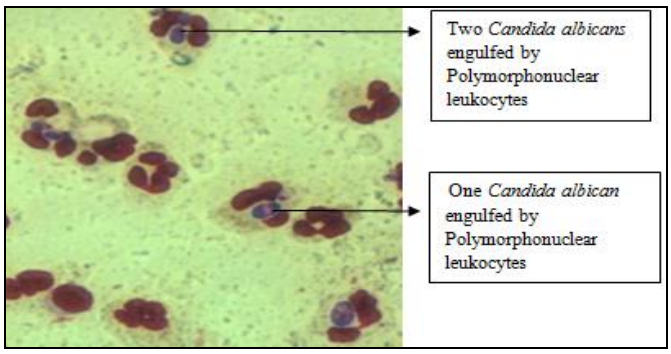

FIG. 2: THE *CANDIDA ALBICANS* ENGULFED BY POLYMORPHONUCLEAR LEUKOCYTES

TABLE 3: RESULT OF PERCENT PHAGOCYTOSIS FOR EACH CONCENTRATION OF *PUTRANJIVA ROXBURGHII* WALL.

Vol. no.	MEM	*T. cordifolia* 400(µg/cm^3)	Concentrations of *Putranjiva roxburghii* Wall. (µg/cm^3)					
			P50	P100	P200	P400	P600	P800
1	25	36	25	27	28	31	34	34
2	29	34	26	28	30	30	33	32
3	30	34	22	25	28	29	30	32
4	27	37	22	24	27	30	31	37
5	28	35	23	25	28	30	32	34
6	27	34	24	26	29	32	34	35
Mean	27.67	35.00	23.67	25.83	28.33	30.33	32.33	34.00
SD	1.75	1.26	1.63	1.47	1.03	1.03	1.63	1.90

Total hundred polymorphonuclear cells were scanned on each of the slide which included polymorphonuclear cells showing no phagocytosis as well as those with ingested *Candida albicans.* The number of *Candida albicans* engulfed (0, 1, 2, 3) in each phagocytic polymprphonuclear cells were counted. **Fig. 2** shows the *Candida albicans* engulfed by polymorphonuclear leukocytes treated with leaf powder of *Putranjiva roxburghii* Wall. The percent phagocytosis and phagocytic index obtained for each selected volunteer is given in **Table 3** and **4**.

TABLE 4: RESULT OF PHAGOCYTIC INDEX FOR EACH CONCENTRATION OF *PUTRANJIVA ROXBURGHII* WALL.

Vol. no	MEM	*T. cordifolia* 400 (µg/cm^3)	Concentrations of *Putranjiva roxburghii* Wall. (µg/cm^3)					
			P50	P100	P200	P400	P600	P800
1	1.24	1.32	1.04	1.07	1.14	1.19	1.32	1.32
2	1.21	1.34	1.15	1.25	1.27	1.30	1.33	1.34
3	1.27	1.24	1.05	1.16	1.21	1.21	1.20	1.25
4	1.22	1.25	1.14	1.13	1.15	1.27	1.26	1.27
5	1.18	1.29	1.13	1.20	1.25	1.27	1.22	1.35
6	1.15	1.26	1.08	1.15	1.17	1.28	1.38	1.37
Mean	1.21	1.28	1.10	1.16	1.20	1.25	1.29	1.32
SD	0.04	0.04	0.05	0.06	0.05	0.04	0.07	0.05

Estimation of Chemotaxis: [4]

Procedure: About 0.2 g of agarose powder was accurately weighed and suspended in 10.0 cm^3 of sterile distilled water. The agarose solution was then heated and the hot solution was mixed immediately with 1.0 cm^3 of pooled human serum and 9.0 cm^3 of Minimum Essential Media.

The resultant medium was poured immediately on a clean glass slide and the agarose was allowed to set for 30 minutes. After 30 min, three wells were bored of 3 mm diameter each on the agarose slide (using sterile pipette tip). The wells were at a distance of 3.0 mm from each other. On the opposite side of the slide, three spots were marked which correspond to wells bored in agarose. The wells were labeled as C, P and M which stand for chemotactic factor, poly-morphonuclear leukocytes and Minimum Essential Media respectively.

For chemotaxis assay system consisted of 20.0 µL of polymorphonuclear leukocytes (adjusted to 2.5 × 10^5 cells/cm^3 as described earlier.) and 20µL of *Putranjiva roxburghii* Wall. (50-800 µg/cm^3) and the assay system for chemotaxis was prepared as follows. 20.0 µL of adjusted polymorphonuclear leukocytes and 20.0 µL extract of *Putranjiva roxburghii* Wall. (50 µg/cm^3) were mixed together, 20.0 µL of this mixture was added to the central well on the agarose slide which was marked as C.

The outer wells contain 20.0 µL of Minimum Essential Media which was marked as M, and the innermost well contain 20.0 µL of chemotactic factor (Zymosan), which was marked as C. Similar procedure was followed for other concentrations of Putranjiva roxburghii Wall. (100-800 µg/cm^3). All slides were then incubated at 37 °C in a 5% CO2 atmosphere for 2 h.

TABLE 5: RESULT OF CHEMOTACTIC DIFFERENTIAL OBTAINED FOR DIFFERENT CONCENTRATION OF TEST DRUG

Vol. no	MEM	*T. cordifolia* 400(µg/cm^3)	Concentrations of *Putranjiva roxburghii* Wall. (µg/cm^3)					
			P50	P100	P200	P400	P600	P800
1	212	464	204	220	276	300	312	464
2	232	528	216	224	260	292	328	524
3	216	484	212	232	280	296	332	488
4	232	464	236	256	272	336	388	440
5	224	508	208	240	248	276	372	528
6	212	492	224	252	260	348	388	448
Mean	221.33	490.00	216.67	237.33	266.00	308.00	353.33	482.00
SD	9.35	25.14	11.71	14.68	12.07	27.83	33.34	37.84

TABLE 6: RESULT OF CHEMOTACTIC INDEX OBTAINED FOR DIFFERENT CONCENTRATION OF TEST DRUG

Concentrations of *Putranjiva roxburghii* Wall. (μg/cm^3)								
Vol. no	**MEM**	**Tc 400**	**P50**	**P100**	**P200**	**P400**	**P600**	**P800**
1	1.28	1.42	1.29	1.31	1.37	1.40	1.41	1.59
2	1.28	1.46	1.29	1.30	1.34	1.37	1.39	1.62
3	1.27	1.40	1.30	1.32	1.36	1.35	1.35	1.48
4	1.30	1.39	1.33	1.33	1.31	1.35	1.36	1.39
5	1.29	1.44	1.28	1.32	1.28	1.29	1.37	1.50
6	1.29	1.41	1.29	1.32	1.29	1.36	1.38	1.40
Mean	1.29	1.42	1.30	1.32	1.33	1.35	1.38	1.50
SD	0.01	0.03	0.02	0.01	0.04	0.04	0.02	0.09

The cells were fixed with methanol for 1 h. The agarose on the slide was the gently removed and the slides were air-dried. The slides were then stained using Giemsa stain (1:8) for 30 min and the slides were washed under gently running tap water. The migration patterns were observed under microscope for spontaneous migration (distance from the central well to Minimum Essential Media) and directional migration *i.e*. Chemotaxis (distance from the central well to the chemotactic factor).

Observations: The percentage phagocytosis in the vehicle control *i.e*. where Minimum Essential Media was added instead of *Putranjiva roxburghii* Wall. was 27.67±1.75. The polymorphonuclear leukocytes subjected with *Tinospora cordifolia* Miers. showed higher percentage phagocytosis (35.00 ± 1.26) when compared to Minimum Essential Media. The polymorphonuclear leukocytes subjected with *Putranjiva roxburghii* Wall. showed comparable percentage phagocytosis to *Tinospora cordifolia* Miers. when polymorphonuclear leukocytes incubated with concentrations of 400 μg/cm^3, 600 μg/cm^3 and 800 μg/cm^3.

The Phagocytic index of polymorphonuclear leukocytes incubated with Minimum Essential Media (control) was found to be 1.21 ± 0.04. Polymorphonuclear leukocytes incubated with *Tinospora cordifolia* Miers. exhibited significant increase in Phagocytic Index (1.28 ± 0.04). The Phagocytic index at 400 μg/cm^3, 600 μg/cm^3 and 800 μg/cm^3, *Putranjiva roxburghii* Wall. increased the phagocytic index significantly when compared to *Tinospora cordifolia* Miers. The chemotactic differential of the Minimum Essential Media (vehicle) was found to be 221.33 ± 9.35. *Tinospora cordifolia* Miers. (positive control) stimulated polymorphonuclear leukocytes shows significant increase (490.00 ± 25.14) in the chemotactic differential when compared to Minimum Essential Media (vehicle). Chemotactic differentials produced by *Putranjiva roxburghii* Wall. were comparable at concentration of400 μg/cm^3, 600 μg/cm^3 and 800 μg/cm^3. The chemotactic index obtained for *Putranjiva roxburghii* Wall. at the concentration of 400 μg/cm^3, 600 μg/cm^3 and 800 μg/cm^3 was comparable with *Tinospora cordifolia* Miers. (positive control).

DISCUSSION: In the present research work immunomodulatory activity of aqueous extract of leaf powder of *Putranjiva roxburghii* Wall. has been evaluated by subjecting the polymorpho-nuclear leukocyte cells isolated from normal healthy volunteers with *Candida albicans* and Zymosan (chemotactic agent). The maximum immunomodulatory activity of *Tinospora cordifolia* Miers. has been reported in a literature [2] for 400 μg/cm^3, whereas *Putranjiva roxburghii* Wall. increased the percentage phagocytosis in a concentration dependent manner.

The lowest concentrations of *Putranjiva roxburghii* Wall. *i.e*. 50 μg/cm^3 shows lower percentage phagocytosis as compared to Minimum Essential Media (vehicle) while polymorphonuclear leukocytes when incubated with concentrations 400 μg/cm^3, 600 μg/cm^3 and 800 μg/cm^3 of *Putranjiva roxburghii* Wall. showed comparable percentage phagocytosis to *Tinospora cordifolia* Miers.

Cells incubated with the different concentrations of *Putranjiva roxburghii* Wall. showed a concentration dependent increase in chemotactic differential. All the values of *Putranjiva roxburghii* Wall. were significantly higher when compared to the Minimum Essential Media (vehicle) except for 50 μg/cm^3, which was comparable to Minimum Essential Media (vehicle).

The chemotactic index obtained for *Putranjiva roxburghii* Wall. at the concentration of 800 $\mu g/cm^3$ was found to be 1.50 ± 0.09 which was greater than the chemotactic index obtained for *Tinospora cordifolia* Miers. (1.42 ± 0.03).

CONCLUSION: From the results obtained, it can be concluded that the aqueous extract of the leaf powder of *Putranjiva roxburghii* Wall. exhibited significant effect on phagocytosis by human neutrophils and chemotactic locomotion of neutrophils. Thus the leaf powder of *Putranjiva roxburghii* Wall. can be recommended for use in certain herbal formulations with immune enhancing activity.

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CONFLICT OF INTEREST: Author affirm that they do not have any conflict of interest.

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