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IN-VITRO IMMUNOMODULATORY EFFECTS OF ISOLATED BARTOGENIC ACID FROM *BARRINGTONIA RACEMOSA* ROXB.

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ABSTRACT

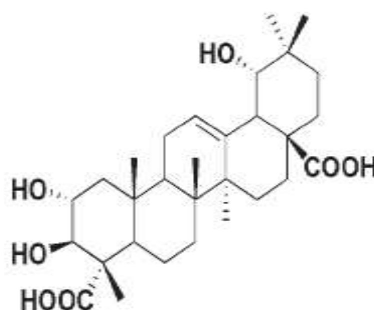
Bartogenic acid is mainly present in the bark and seeds of *Barringtonia racemosa.* Recent reports claim that *B. racemosa* bark extract possesses significant antinoceiceptive and anti-tumor activity. This effect has been proposed to be related to cytotoxic and immunomodulatory effects of this plant. In present study isolated Bartogenic acid from powdered fruits of *B. racemosa* was evaluated in *in-vitro* models of immunity *viz.* Nitro Blue Tetrazolium reduction assay (NBT), PMN cell Phagocytosis assay, Intracellular killing of *Candida albicans* by PMN cells, PMN cell Chemotaxis assay. The bartogenic acid showed stimulatory activity on PMN cell chemotaxis. It is proposed that the alterations in the oxidative processes caused by bartogenic acid which were evident in phagocytic and intracellular killing activities of PMN cells contribute to the increased chemotaxis of PMN cells. It was found that isolated bartogenic acid shows a potent immunostimulant activity in *in vitro* models.

KEYWORDS: *Barringtonia racemosa*, Immunomodulation, PMN cells, Chemotaxis, Phagocytosis.

INTRODUCTION

The modulation of immune response by using medicinal plant products as a possible therapeutic mean has become a subject of scientific investigation. Some of these plants like *Tinospora cordifolia* have been clinically proved to be effective in infectious and febrile conditions. The main factors that make natural products attractive candidates for human use include their ease of availability, cost effectiveness and presumed safety.[1] *Barringtonia*

racemosa (*B. racemosa*) (Barringtoniaceae) fruit pulp had been in use as a fish poison while other parts like bark are used in the folk medicines as a treatment of diarrhea, asthma, coughs, jaundice as well as fever.[2,3] Bartogenic acid is a triterpene dicarboxylic acid. Its structure was established as 2α,3β,19α-trihydroxyolean-12-ene-24,28-dioic acid.[4] Bartogenic acid is mainly present in the bark and seeds of *Barringtonia racemosa.*[5] Recent reports claim that *B. racemosa* bark extract possesses significant antinoceiceptive and anti-tumor activity.[3,6] This effect has been proposed to be related to cytotoxic and immunomodulatory effects of this plant. However, systematic evaluation of different fractions of fruit extract of plant *B. racemosa* has not been reported. In present investigation, isolated bartogenic acid from fruits of *B. racemosa* has been evaluated for its *in vitro* immunomodulatory effect.



Bartogenic Acid (2α,3□,19α -trihydroxyolean-12-en-24,28-dioic acid)

MATERIALS AND METHODS

Materials:

Formyl-N-Methyl-Leucyl-Phenylalanine (FMLP), DMSO HYBRI-MAX, Sodium Deoxycholate were purchased from Sigma- Aldrich; RPMI 1640, MEM Eagle, Nitroblue tetrazolium), May-Grunwald stain , Giemsa stain, Agarose Electrophoresis grade, Fetal Bovine Serum were purchased from Himedias Laboratories, India whereas, Dextran T-500 was obtained from Pharmacosmos Denmark. The *Candida albicans* culture (ATCC 10231) was obtained from National Chemical Laboratory, Pune (India).

Marker Compound for Bartogenic acid was issued by department of Natural Product Chemistry, Indian Institute of Chemical Technology (IICT), Hyderabad.

Drug preparation

The isolated compound bartogenic acid for *in vitro* assays was dissolved in dimethylsulfoxide (DMSO) and serial dilutions were prepared in RPMI 1640 in such a way that the final

concentration of DMSO in the assay medium did not exceed 1% v/v. The range of concentrations studied in *in vitro* assays was 50 µg/ml to 25ng/ml.

Isolation and preparation of human PMN Cells

PMN cells were separated from the fresh heparinized blood samples from human volunteers by dextran (T-500) sedimentation method as reported elsewhere.[7] The heparinized blood was mixed with equal amount of 1.75% Dextran solution in saline and allowed to sediment at room temperature for 45 minutes. The supernatant was collected and washed thrice with sterile phosphate buffered saline (PBS). The resultant pellet of cells was suspended in RPMI-1640 medium containing 10% Fetal calf serum (FCS). The PMN cell count determined microscopically using Neubaur's chambers and the cell count for different assays was adjusted with RPMI-1640 medium.

Assessment of the non Specific Immune Function *in vitro*

Phagocytosis of *candida albicans* by PMN cells

For the phagocytosis assay, 100 µl of PMN cell suspension was mixed with 100 µl of *Candida* cell suspension, 100 µl of test drug solutions of different concentration in RPMI-1640 and 100 µl of RPMI 1640 containing FCS. The final concentrations of bartogenic acid in the assay mixtures ranged from 25 ng/ml to50 µg/ml. In the positive control assay unit, instead of bartogenic acid, 100 µl of serum derived from the same human volunteer was taken. The assay mixture was incubated at 37^0C and 5% CO_2 for 30 minutes in CO_2 incubator.[8,9] 20 µl of sample from the above mixture was taken and smear was prepared on microscopic slide. The smear was air dried, fixed with methanol for 5 minutes and stained with May-grunwald's and Giemsa. These slides were observed under oil immersion microscopy and average number of engulfed *Candida* associated with PMN cells was determined.[9]

Intracellular Killing of *Candida albicans* by PMN cells

Solutions are prepared as mentioned in Phagocytosis assay. After 60 minutes of incubation 0.1 ml of 2.5% sodium deoxycholate (pH 8.7) was added to each tube. At this concentration, deoxycholate causes immediate lysis of the blood cells without damage to the Candida cells. Methylene blue, 0.01% in distilled water, was then added to achieve a final volume of 4 to 5 ml. The *Candida* cell suspensions were centrifuged at 1,100 g for 15 min at 4^0 C in cooling centrifuge and resuspended in about 0.5 ml of the residual supernatant fluid. The tubes were

kept in ice water bath immediately observed microscopically for percentage of live *Candida* cells.[10]

Nitroblue tetrazolium (NBT) reduction by PMN Cells

In a flat bottom 96-well plate 20µl of the PMN cell suspension (1×10^6 cells/ml) and 40µl of RPMI 1640 were pipetted. 20µl Bartogenic acid solutions in RPMI-1640 were pipetted into these wells to get the final concentrations of bartogenic acid from 25 ng/ml to 50 µg/ml. After incubation for 24 hours at 37°C in humidified 5% CO_2 in a CO_2 incubator, 20µl of pooled human serum and 20µl of NBT solution in PBS (1.5mg/ml) were added to the mixture.[11] The plate was further incubated for an hour. After incubation, the cells were washed four times with 200 µl methanol and the resultant residue was air dried. After this to each well, 120µl of 2M KOH and 140µl of DMSO were added. The optical densities of contents of wells were measured at 570 nm using microplate spectrophotometer.[12]

***In vitro* PMN cell Chemotaxis**

Microscopic slides of 50 X 75 mm dimensions were loaded with 6 ml mixture of heat dissolved agarose in sterile and pyrogen free water (concentration 2.4%) and Minimum essential medium in 1:1 proportion. After proper settling at 4^0C, wells of 4 mm diameter were bored in the settled agarose in such a way the distance between the wells was exactly 5 mm. The wells were arranged in such a way that the central well containing FMLP solution was surrounded by four wells containing PMN cell suspensions along with different concentrations of bartogenic acid.[13,14] To the central wells, 20 ml of FMLP solution ($1X\ 10^{-8}$) was added while to the surrounding wells 10 µl PMN cell suspension and 10 µl drug solution (in RPMI-1640) were added. The plates were incubated in CO_2 incubator for 2 hours followed by fixing of the cells in methanol and formaldehyde for 30 min each. The slides were stained with Giemsa stain and observed under 10X power of microscope for chemotaxis. The chemotactic index was determined by subtraction of the distance traversed by cells towards FMLP and random kinesis on the opposite front.[15]

RESULTS AND DISCUSSION

Statistical analysis

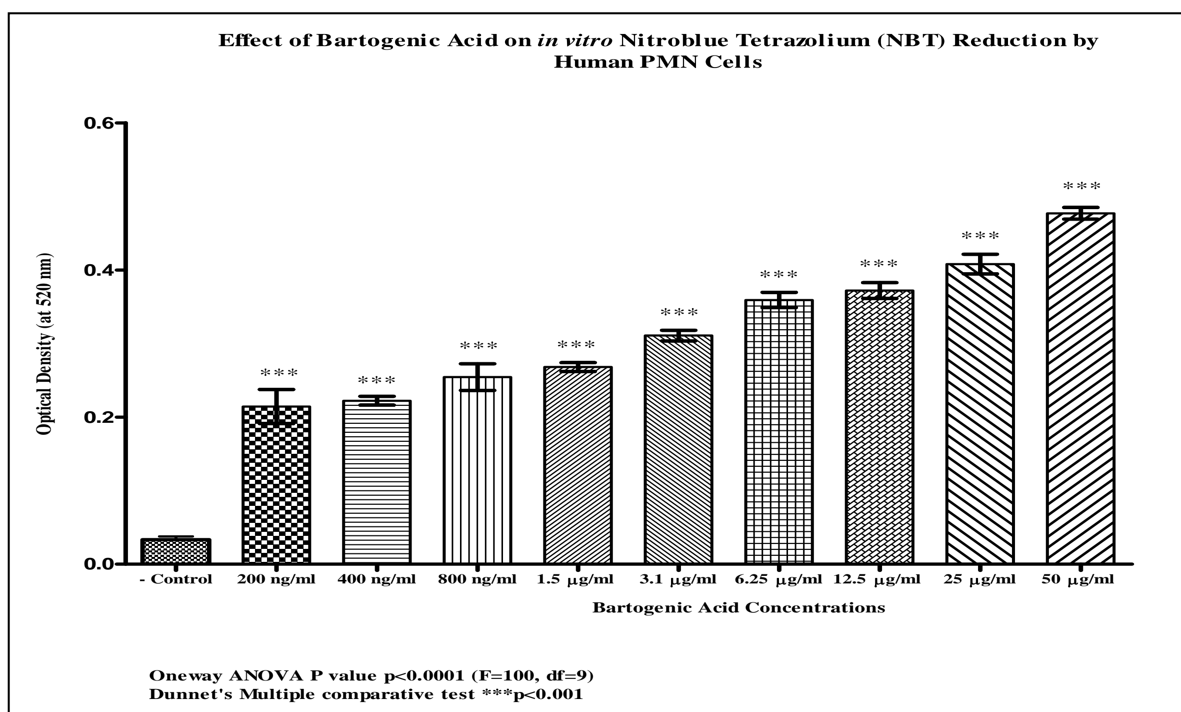
The statistical analysis was done using One Way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test. Results with $p < 0.05$ were considered statistically significant. Data has been expressed as mean ± S.E.M.

Effects on Innate Immune Response

a. Effect of Bartogenic Acid on *in vitro* Nitroblue Tetrazolium (NBT) Reduction by Human Cells

PMN cells were incubated in presence of different concentrations of bartogenic acid with NBT stain. At the end of incubation period a sample of PMN cells were washed with methanol and NBT were dissolved in 2M KOH and DMSO. The absorbance were taken at 570 nm results have been stated as optical Density of reduced NBT.

All the doses of bartogenic acid showed the dose dependent increase in NBT reduction by human PMN cells. All the doses showed significant increase in the NBT reduction 200 ng/ml to 50 µg/ml (p<0.001) as compare to negative control.

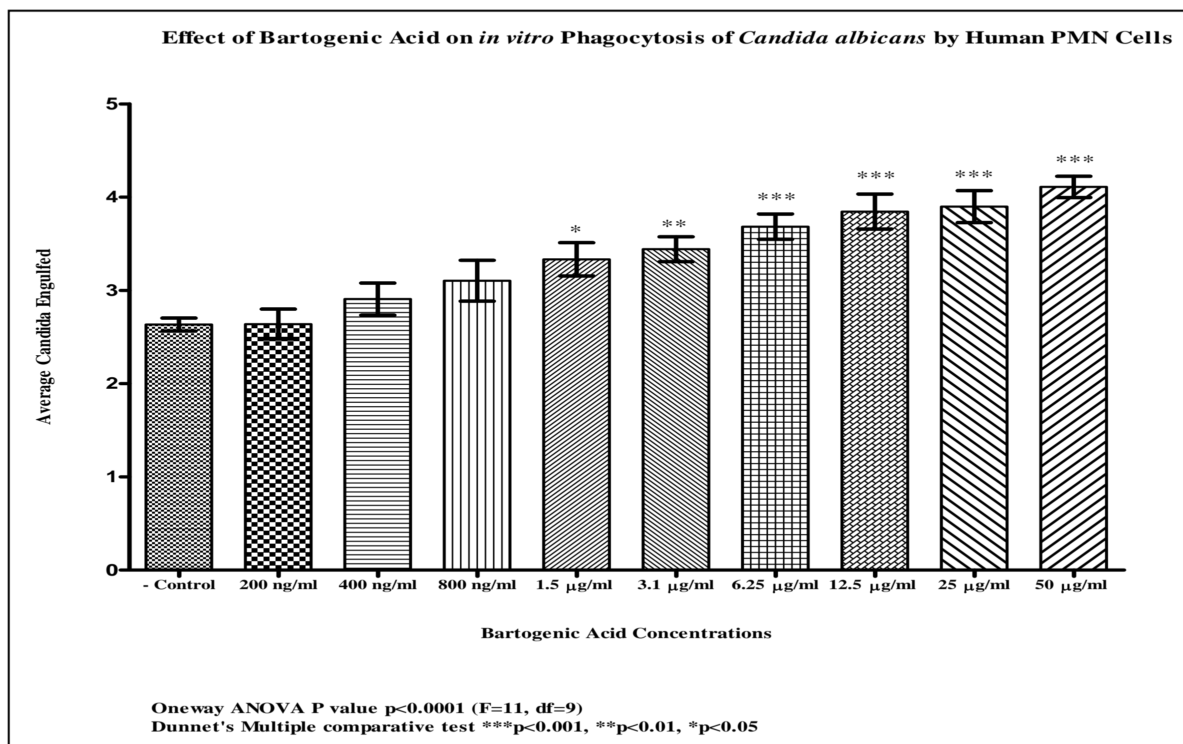


Mean	0.033	0.21	0.22	0.25	0.27	0.31	0.36	0.37	0.41	0.48
Std.Deviation	0.011	0.057	0.015	0.044	0.014	0.018	0.025	0.026	0.033	0.02
Std. Error	0.0046	0.023	0.006	0.018	0.0059	0.0072	0.01	0.011	0.013	0.008

b. Effect of Bartogenic Acid on in vitro Phagocytosis of *Candida albicans* by Human PMN cells

Effect of different concentrations of bartogenic acid on the PMN cell Phagocytic activity was estimated by determining average number of *Candida* cells associated with or engulfed by PMN cells after co-incubation of PMN cells, different concentrations of extracts ranging from 200 ng/ml to 50 µg/ml along with the optimized unicellular *Candida albicans* cells. The

results have been expressed as 'Minimum Particle Number' (MPN), the average number of *Candida* cells associated with PMN cells. Bartogenic acid showed stimulatory effect on phagocytosis; this effect was reduced with decreasing concentrations of bartogenic acid. This reveals a dose dependent stimulatory effect of bartogenic acid on phagocytosis. The significant increase in the phagocytosis were found at the doses of 1.5 µg/ml ($p<0.05$), 3.1 µg/ml ($p<0.01$) and at doses of 6.25, 12.5, 25, 50 µg/ml ($p<0.001$) as compare to negative control.

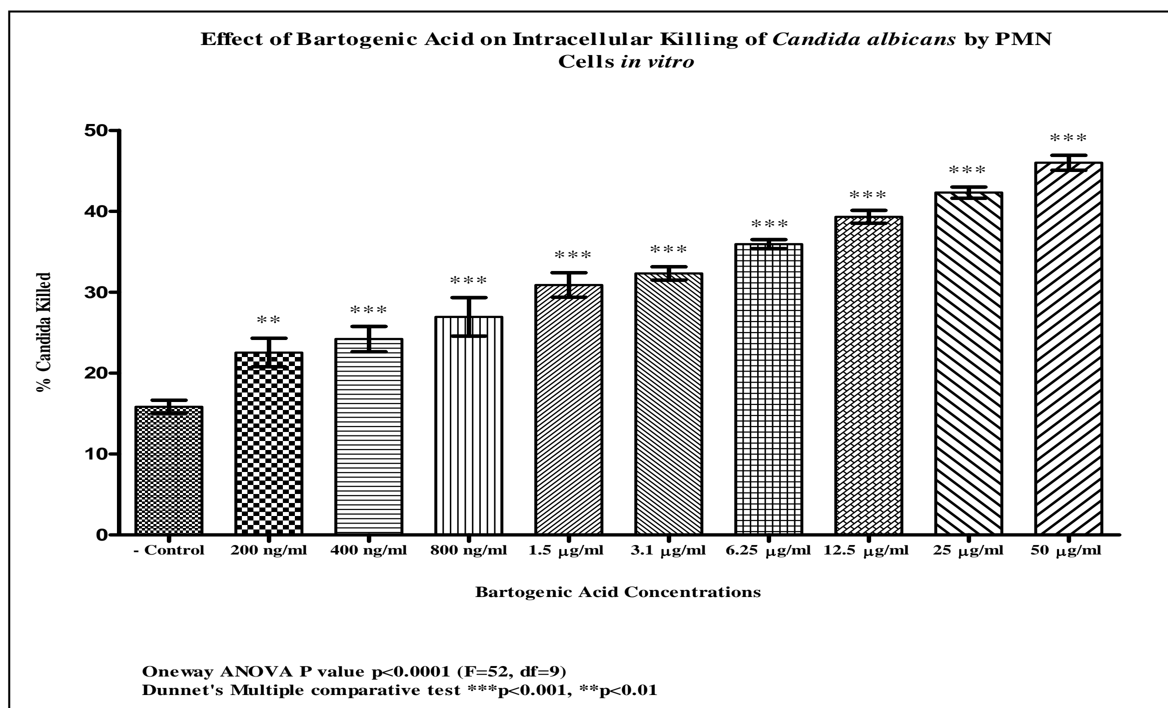


Mean	2.6	2.6	2.9	3.1	3.3	3.4	3.7	3.8	3.9	4.1
Std.Deviation	0.17	0.4	0.42	0.54	0.44	0.33	0.33	0.46	0.42	0.28
Std. Error	0.07	0.16	0.17	0.22	0.18	0.13	0.14	0.19	0.17	0.11

C. Effect of Bartogenic Acid on in vitro Intracellular Killing of *Candida albicans* by Human PMN cells

Effect of different concentrations of bartogenic acid on the Intracellular killing of *Candida albicans* by human PMN was estimated by determining % of *Candida* killed by PMN cells after co-incubation of PMN cells, different concentrations of extracts ranging from 200 ng/ml to 50 µg/ml along with the optimized unicellular *Candida albicans* cells. The results have been expressed as 'Percent Candida Killed' by PMN cells.

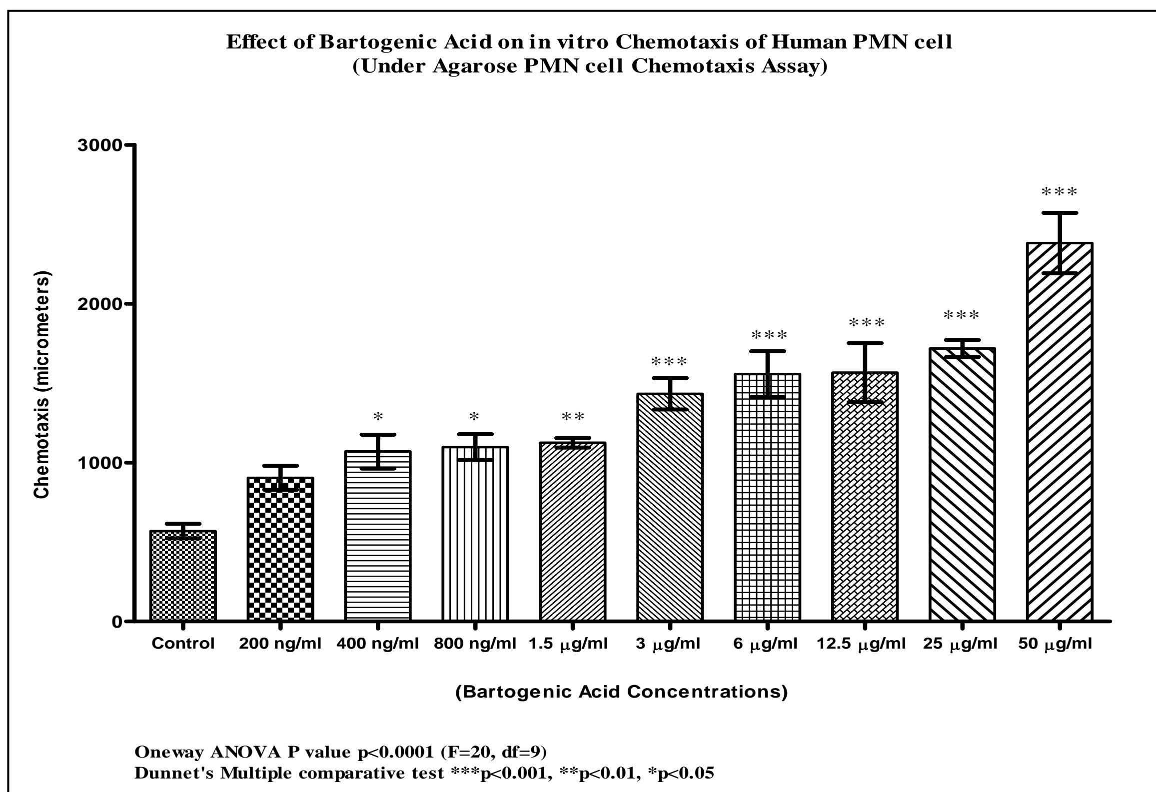
Bartogenic acid showed stimulatory effect on intracellular killing of *Candida albicans* by human PMN cells; this effect was increased with increasing concentrations of bartogenic acid. This reveals a dose dependent stimulatory effect of bartogenic acid on intracellular killing. The significant increase in the intracellular killing of *Candida albicans* were found at the doses of 200ng/ml (p<0.01), 400, 800 ng/ml and 1.5, 3.1, 6.25, 12.5, 25, 50 µg/ml (p<0.001) as compare to negative control.



Mean	16	23	24	27	31	32	36	39	42	46
Std.Deviation	2	4.3	3.8	5.8	3.7	2	1.4	2	1.7	2.3
Std. Error	0.81	1.8	1.6	2.4	1.5	0.82	0.56	0.8	0.69	0.93

D. Effect of Bartogenic Acid on in vitro Chemotaxis of Human PMN cell (Under Agarose PMN cell Chemotaxis Assay)

The effect on PMN cell chemotaxis was evaluated by under agarose method where the chemotaxis was induced by FMLP. The migration of PMN cells towards the FMLP were taken as the parameter. The migrations were measured in µm under 10X lense. Bartogenic acid was found to increase the chemotaxis in a concentration dependent manner. The significant increase in the chemotaxis were observed at different concentration i.e. 400, 800 ng/ml (p<0.05), 1.5 µg/ml (<0.01) and 3.1, 6.25, 12.5, 25, 50 µg/ml (p<0.001) as compare to negative control.



Mean	568	904	1069	1098	1125	1433	1557	1566	1718	2381
Std.Deviation	113	185	260	198	73	242	352	454	132	465
Std. Error	46	75	106	81	30	99	144	186	54	190

Hence, it is proposed that bartogenic acid has very potent stimulatory effects on intracellular oxidative processes.

Bartogenic acid showed significant increase in the phagocytic activity in a concentration dependent manner. The processes by which PMN cells ingest microorganisms were explored using *Candida albicans*. In case of intracellular killing of *Candida albicans* the Bartogenic acid showed the same effect as that in Phagocytosis.

CONCLUSION

The bartogenic acid showed stimulatory activity on PMN cell chemotaxis. Cell migration (chemotaxis) was found to increase in a concentration dependent manner at all the concentrations tested. It is proposed that the alterations in the oxidative processes caused by bartogenic acid which were evident in phagocytic and intracellular killing activities of PMN cells contribute to the increased chemotaxis of PMN cells. However, actual mechanism of this effect cannot be clearly delineated at this stage. Further study involving evaluation of

effects of bartogenic acid on individual kinases and other enzymes is needed for confirmation of the mechanism of action.

REFERENCES

1. Mediratta P K, Sharma K K, Singh S. Evaluation of immunomodulatory potential of Ocimum sanctum seed oil and its possible mechanism of action. Journal of Ethnopharmacol., 2002; 80: 15-20.
2. Khan S, Jabbar A, Hasan C M, Rashid M A,. Antibacterial activity of Barringtonia racemosa. Fitoterapia., 2001; 72: 162-164.
3. Ratnasooriya W D, Deraniyagala S A, Goonasekara C L. Antinociceptive effect and toxicological study of the aqueous bark extract of Barringtonia racemosa on rats. Journal of Ethnopharmacol., 2003; 86: 21–26.
4. Mallavarapu G R, Rao G S R S, Prasanna S, Kumar V P S. Bartogenic acid, a new triterpene acid from Barringtonia speciosa. Phytochemistry 1980; 20: 333-334.
5. Rao J M, Gowri P M, Tiwari A K, Ali A Z,. Inhibition of α-Glucosidase and Amylase by Bartogenic Acid Isolated from Barringtonia racemosa Roxb. Seeds. Phytotherapy Research., 2007; 21(8): 796-799.
6. Thomas TJ, Panikkar B, Subramoniam A, Nair MK, Panikkar KR. Antitumour property and toxicity of Barringtonia racemosa Roxb. seed extract in mic.e Journal of Ethnopharmacology., 2002; 82: 223-227.
7. Lunardi F, Hermenio J, Lima C, Assreuy J. Comparative study of respiratory burst induced by phorbol ester and zymosan in human granulocytes.Clinical Biochemistry., 2006; 39: 78–85.
8. Bin-Hafeez B, Haque R, Parvez S, Pandey S, Sayeed I, Raisuddin S. Immunomodulatory effects of fenugreek (Trigonella foenum graecum L.) extract in mice. International Immunophrmacology., 2003; 3: 257-265.
9. Gabhe S Y, Tatke P A, Khan T A. Evaluation of the Immunomodulatory activity of methanolic extract of ficus benghalensis roots in rats. Indian J Pharmacology., 2006; 38(4): 271-275.
10. Lehrer R I, Cline M J,. Interaction of C.albicans with human leucocytes and serum. Bacteriology., 1969; 98(3): 996-1006.
11. Petra R, Gabriele W, Connie J, Bengt R, Egbert H, Influence of modified natural and synthetic surfactant preparations on bacterial killing by polymorphonuclear leucocytes. Immunobiology., 2004; 209: 609–617.

12. Manosroi A, Saraphanchotiwitthaya A, Manosroi J. Effect of Pouteria cambodiana on in vitro immunomodulatory activity of mouse immune system. Fitoterapia., 2006; 77: 189-193.
13. Nash D K N, Tonellato P, Swiersz M, Abramoff P. Assessment of Chemokinetic behavior of inflammatory lung macrophages in a linear under-agarose assay. Journal of Leuko Bio., 1990; 48: 297-305.
14. Cotter M J, Norman K E, Hellewell P G, Ridger V C. A novel method for isolation of Neutrophils from murine blood using negative immunomagnetic separation. American Journal of Pathology., 2001; 159(2): 473-481.
15. Nagata T, Kansha M, Takahashi S, Takahashi I.. Propofol inhibits fMLP stimulated phosporylation of p-42 mitogen activated protein kinase and chemotaxis in human neutrophils. British Journal of Anesthesia., 2001; 86(6): 853-858.